INDEX

VOL 7. FASC 1–4 1969

SUPPLEMENTUM

Acta path microbiol scandinav 75 1–17 1969

Ullevål Hospital Department of Pathology (Head Kristen Arnesen M D)
University of Oslo Oslo Norway

THE DEPOSITS OF FIBRIN AND FIBRIN LIKE MATERIALS IN THE BASAL PLATE OF THE NORMAL HUMAN PLACENTA

By

NARVE MOE

Received 17 v 68

The basal plate in the normal human placenta is composed of two cell layers the foetal cytotrophoblastic shell which lines the intervillous space and which is primarily formed from the cytotrophoblastic cell columns (anchoring villi) (38) and the maternal decidua which forms the parietal part

The morphological distinction between cytotrophoblastic and decidual cells can be difficult In general the decidual cells almost lack basophilia in contrast to the intense basophilia of the trophoblast (9) The interstitial ground substance of the decidua contains argyrophilic fibres (19 38) and shows metachromasia with toluidine blue (39) These are characteristics not shared by the material between the cytotrophoblastic cells However several authors (22 35 36) claim that the basal plate already at an early stage consists only of decidual cells

With advancing gestational age increasing amounts of intercellular deposits are formed between the individual cells of the cytotrophoblastic shell and on both sides of the shell

On the surface of the basal plate towards the intervillous space *Rohr* in 1889 described a layer (Rohr s stria) which he considered to be fibrin Not much attention has been given to the origin of this layer

Rohr s stria gradually merges with a material deposited between the cytotrophoblastic cells This material has been designated by the non committal term fibrinoid (15 38)

The deposit present in the junctional zone between the trophoblast and decidua is called Nitabuch s layer (27) Several authors consider the material here to be true fibrin (5 16 17 22 27) whereas others prefer to call also this deposit fibrinoid (3 14 15 38 42)

Thus the nature of the deposits in the basal plate is not determined With one exception the previous investigations are all light micro

scopical studies Only Wynn (42) made an ultrastructural study and stated that the deposits might arise from degeneration of decidual or trophoblastic cytoplasm or through transformation of the ground substance

The present study is a combined light and electron microscopical investigation of the nature of the different deposits in the basal plate of human placentas of varying gestational age It will be shown that they are not identical in structure and evidence will be presented indicating that their mechanism of formation differs

MATERIAL AND METHODS

Thirty three mature and 26 immature placentas were examined only by light micros opy The immature placentas were obtained from physically healthy woman 8 to 24 weeks pregnant (menstrual age) and removed by curettage or minor caesarean section The pregnancies were interrupted on psychiatric indications

Two mature and 3 immature placentas of 10 13 and 24 weeks gestation were examined by both light and electron micros opy

Light Microscopy

Immediately after delivery or operation the placentas were gently rinsed in 0 9 per cent NaCl solution and cut in thin slices Specimens were taken from the central and the peripheral parts of the basal plate and fixed in buffered formaline Zenker s or Helly s solution dehydrated and embedded in paraffin The following stains were used

1) Haematoxylin azo phloxine (HP)
2) Lendrum s Martius s arlet blue method (MSB) (21)
3) Mallory s phosphotungstic acid haematoxylin method (PTAH)
4) Gomori s silver method for argyrophilic fibrils
5) Toluidine blue (0 1 per cent)

Electron Microscopy

Tissue blocks from the basal part of the placenta were removed immediately after delivery or operation and fixed for 1½–2 hours in chilled isotonic 1½ per cent glutaraldehyde in M/20 phosphate buffer (pH 7 4) postfixed for 1½ hour in 1 per cent isotonic osmic tetroxide (C) and embedded in Epon 812 One micron thick sections were cut on a Huxley ultramicrotome (Cambridge Inst Co) and stained with 0 2 per cent toluidine blue for orientation by light microscopy Suitable areas were selected and ultrathin sections stained with uranyl acetate and lead citrate (29) The preparations were examined in a Zeiss EM 9 electron microscope Serial photographs were mounted on cloth to facilitate localization

RESULTS

The basal plate of the normal human placenta showed considerable variation in thickness in relative quantity of foetal and maternal elements and in localization and amount of deposits

Cellular Elements

The basal plate was thinner in placenta at term than in the immature placenta In the latter scattered syncytial elements lined parts of the intervillous space and extended occasionally into deeper parts of the

basal plate With increasing gestational age only a few flattened endothelial like cells were seen on the surface (Figs 1 11 and 16)

A nearly continuous layer of cytotrophoblastic cells was present in the basal plate both in immature and mature placentas The differentiation between cells belonging to the cytotrophoblastic shell and decidual cells was based mainly on staining and structural properties By light microscopy the cytotrophoblastic cells had basophilic cytoplasm and large nuclei with a coarse chromatin net (Figs 1 and 16) In the immature placenta the cytotrophoblastic shell showed direct continuity with cytotrophoblastic cell columns (anchoring villi) and the cells of those two structures were similar The cytotrophoblastic shell was particularly well developed close to the anchoring villi In areas distant from the anchoring villi the layer of cytotrophoblastic cells was occasionally interrupted (Fig 1)

The identification of decidual cells was based upon the weak cytoplasmic basophilia and small nuclei with evenly distributed chromatin (Figs 1 and 16) and the presence of pericellular argyrophilic fibres (Fig 2) Further the interstitial ground substance showed metachromasia with toluidine blue The intercellular material of the cytotrophoblastic shell stained blue with this stain

By electron microscopy orientation within the basal plate could be difficult Sections were only used if characteristic structures or border zones could be definitely identified such as Nitabuch s layer outlined by decidual cells with collagen fibrils interstitially or Rohr s stria towards the intervillous space

Ultrastructurally the cytotrophoblastic cells varied considerably in size at all gestational stages In a given section a cell was only rarely completely surrounded by intercellular material parts of the peripheral membrane were in intimate contact with neighbouring cells often displaying desmosomes (Figs 5 and 14) The cells contained well developed granular endoplasmic reticulum and free ribosomes (Fig 6) and the mitochondria were of medium size (Fig 3) However in the mature placenta many cells had relatively few organelles and the cytoplasm was more electron dense (Fig 5)

In the decidua the cells also varied in size In addition they showed a variation in shape Some of them were spherical others had more irregular outlines with *pseudopods In between fibroblast like cells* were seen Occasionally collagen fibres seemed to be associated with the cell (Fig 4) The cells in the decidua were usually not in mutual contact and desmosomes were not observed Granular endoplasmic reticulum and free ribosomes were less prominent and the mitochondria smaller than in the cytotrophoblastic cells (Fig 4)

In both cell types large osmiophilic bodies probably lipid (Fig 7) and lysosome like bodies (Figs 3 and 5) were encountered Glycogen granules were infrequent

All cytotrophoblastic cells (Fig 7) and occasionally the decidual


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cells as well (Fig 4) displayed signs of secretory activity A prominent Golgi complex surrounded by small vacuoles The vacuoles were limited by a smooth surfaced single unit membrane and contained a finely granular material of low electron density (Fig 6)

Rohr s Stria

By light microscopy Rohr s stria in the immature placenta appeared as a discontinuous layer of extracellular material with fibrillar appearance covering in part the surface of the basal plate towards the intervillous space (Figs 8 9 and 10) usually in association with cells of cytotrophoblastic type In areas where the basal plate consisted of decidual cells only Rohr s stria was mostly absent By MSB stain Rohr s stria took a bright red colour (Fig 8) By PTAH a bluish black colour was obtained (Fig 9) Both reactions suggest that fibrin is a major component of the deposits Blood platelets and a few leucocytes and erythrocytes were frequently seen in association with the fibrillar material *At times the deposit had the appearance of a platelet aggregate* with fibrin (Fig 10)

In the mature placenta Rohr s stria was more constantly present often forming a compact and nearly continuous layer covering even areas devoid of cytotrophoblastic cells As in the immature placenta the stria stained red with MSB and bluish black with PTAH Towards the intervillous space endothelial like cells covered parts of the surface Particularly in areas where the endothelial like cells were missing small projecting masses of platelets and fibrin were encountered on top of the stria (Fig 11)

Electron microscopy of Rohr s stria revealed a network of fibrillar material arranged in thick bundles of high electron density Occasionally the individual strands of the net displayed a cross striation with a periodicity of about 230 Å suggestive of fibrin (Fig 12) In the meshes of the network blood platelets cellular debris and an occasio

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Figs 1–4

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Fig 4 Placenta at 13 k P t n cf l cidu l c ll Th cyt plasm contains mitoch ndria f m ll c C lgi omplex and granular endoplasmic reti culum Coll g n fib re l c ted n the interstitial space (arrows) × 16 500


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In the mature placenta Rohr's stria was more constantly present, often forming a compact and nearly continuous layer covering even areas devoid of cytotrophoblastic cells. As in the immature placenta the stria stained red with MSB and bluish black with PTAH. Towards the intervillous space endothelial like cells covered parts of the surface. Particularly in areas where the endothelial like cells were missing, small projecting masses of platelets and fibrin were encountered on top of the stria (Fig 11).

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Fig 2 Placenta at term. At lower half decidua with interstitial argyrophilic connective tissue fibres. At upper half cytotrophoblastic shell. Gomori's silver method × 500

Fig 3 Placenta at 12 week. Portion of cytotrophoblast from superficial part of cytotrophoblastic shell. The cytoplasm contains mitochondria of medium size as well as Golgi complex, empty vacuole (arrow) and lysosome like bodies. Intercellularly granular masses, filaments and cell debris are seen × 16,500

Fig 4 Placenta at 13 week. Portion of decidual cell. The cytoplasm contains mitochondria of small size, Golgi complex and granular endoplasmic reticulum. Colla[illegible] fib[illegible] located in the interstitial space (arrows) × 16,500


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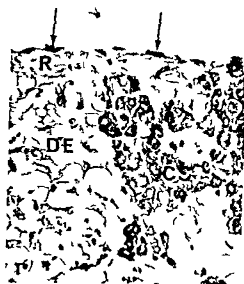
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nai leucocyte were found Mostly the platelets showed more or less complete loss of organelles

Intercellular Deposits within the Cytotrophoblastic Shell

The cells of the cytotrophoblastic shell were often arranged in clusters In other areas the individual cells were separated by a substantial amount of intercellular deposit (Fig 8) which seemed to increase with advancing pregnancy By light microscopy the deposit had usually a granular structure but in some areas it was more hyaline By MSB the deposit stained either red or blue (Figs 8 and 11) PTAH gave it mostly a reddish brown colour with occasional small bluish black specks (Fig 9) These reactions suggest the presence of some fibrin but not everywhere throughout the deposits

Electron microscopy showed that the intercellular deposit within the cytotrophoblastic shell was predominantly composed of a finely granular material of moderate or low electron density mixed with cellular debris (Figs 5 6 13 and 15) The granular material had an appearance like the material found within the vacuoles surrounding the Golgi complex (Fig 6) Occasionally granular material filled gaps in the peripheral cell membrane of the cytotrophoblastic cells creating direct continuity between the intercellular deposit and the cytoplasm (Fig 13) Within the granular material fine filaments were often observed (Figs 5 and 6) Fibrils arranged in thick bundles showing a higher electron density than the granular material and with a periodicity indicative of fibrin were encountered in the intercellular deposit at all ages examined However such fibres were found only in the superficial parts of the shell in the immature placentas (Fig 14) whereas it occurred throughout the shell in the mature ones (Fig 15)

The Junctional Zone (Nitabuch s Layer)

At the junctional zone between foetal and maternal tissues the deposited material (Nitabuch s layer) gave the staining reactions typical

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Figs 5 7

Fig 5 Placenta at term Cytotroph blastic shell At lower half portions [illegible] cells with dense cytoplasm and few organelles At upper half a part of [illegible] tr ph blastic cell with granular end plasmic reticulum numer us [illegible] s mes and a lysosome like body Nucleus at upper right Granul [illegible] filament us masses of moderate ele tron density fill up the intercellular space together with cellular debris × 18 000

Fig 6 Placenta at 12 weeks Cytotroph blastic cells with prominent end [illegible] reticulum and small vacu [illegible] containing a granular material [illegible] density (arr ws) In the intercellular space granular dep it [illegible] appearance i seen × 31 500

Fig 7 Placenta at 13 weeks Cyt troph blastic shell The cell contains [illegible] plex (arrows) and many small vesicles with medium ele tron den e mat [illegible] in relati n to Golgi complex Mito hondria granular end plasmic reti [illegible] and a lipid body are seen × 18 000


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of fibrin by both the MSB (red) (Figs 8 and 10) and the PTAH (bluish black) (Fig 9) methods In the immature stages the structure of the deposits was fibrillar and granular In the mature placenta the deposit was often hyaline In areas where the cytotrophoblastic shell was thin or lacking Nitabuch s layer and Rohr s stria fused In some areas Nitabuch s layer was interrupted and the decidual cells appeared to come in contact with the cytotrophoblastic cells (Fig 16) Scattered trophoblastic cells were occasionally found between the decidual cells but there was no massive invasion of cytotrophoblastic cells into the decidua in areas where Nitabuch s layer was absent

Electron microscopy of the immature placenta showed layers of preserved trophoblast and decidual cells usually separated by a thin zone of altered cells cellular debris occasionally neutrophil leucocytes and granular masses containing scattered bundles of fibrils in which occasionally a cross striation of 230 Å characteristic of fibrin could be recognized (Fig 17)

In the mature placenta Nitabuch s layer showed a substantial increase in thickness The ultrastructure of the deposits was essentially the same as in the immature stage Large amounts of compact moderately electron dense granular to filamentous masses were often the main constituent (Fig 18)

The Interstitial Material of the Decidua

By light microscopy the MSB and PTAH preparations showed no areas which gave a positive stain

Electron microscopy revealed the interstitial material to be collagen fibrils granular material and cellular debris Fibrils with the characteristics of fibrin were encountered adjacent to the junctional zone (Fig 19) whereas such fibrils were not found in the remaining part of the decidua

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Figs 8 11

Fig 8 Placenta at 17 weeks Basal plate with Rohr s stria cyt trophoblastic shell Nitabuch s layer and decidua Positive (red) staining appears dark grey Rohr s stria and Nitabuch s layer give a positive stain Traces of positive material are found in the intercellular deposits of shell (arrows) MSB × 200

Fig 9 Placenta at 17 weeks Parallel section of that in Fig 8 Positive stain (bluish black) appears black PTAH × 200

Fig 10 Placenta at 8 weeks Basal plate with Rohr s stria cytotr phoblastic shell and Nitabuch s layer Rohr s stria Nitabuchs layer and some of the intercellular material of the shell give a positive stain which appears dark grey A large platelet aggregate is situated at the surface of the basal plate MSB × 550

Fig 11 Placenta at term Superficial part of basal plate adjacent to the intervillous space Rohr s stria merges with the intercellular material in the cytotrophoblastic shell which are both stained red with MSB (appears dark grey) A tightl packed platelet aggregate with fibrin is attached to the surface Arrow indicate endothelial like cell MSB × 500


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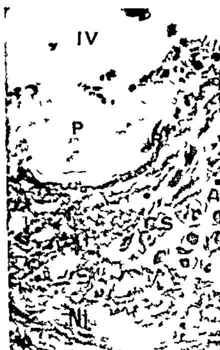
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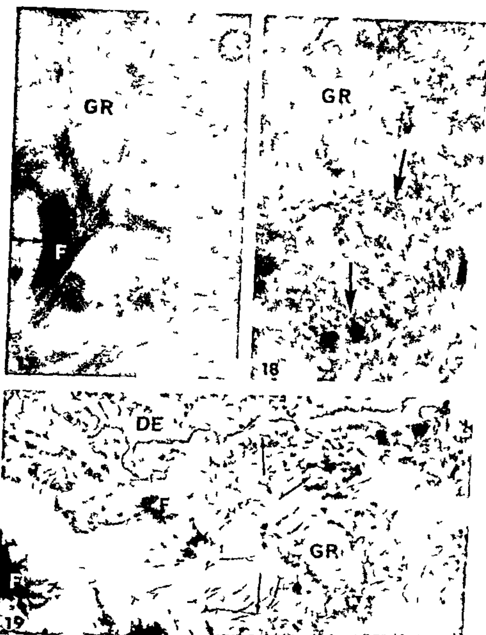
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Fig 17 Placenta at 13 wee[k] Nitabuch la[y]er with cell debri granul[ar] [f]ila ment us mas[s]es and fibrin Arr[o]w indi[c]ates [c]r[o]s[s] [s]triati[o]n × 48 000

Fig 18 Placenta at term Nit buch s layer m [os]tly [com]p[os]ed [o]f granul[ar] [f]ila ment[o]u[s] masses Fibre[s] sugge[s]tive [o]f fibrin (arr[o]w[s]) are al[so] f[oun]d × 18 000

Fig 19 Placenta at 13 week Nitabuch layer a[d]jacent t[o] de[c]idua [co]n[sists o]f granular masses c[o]ll[a]gen fi[b]ril (arr[ow]s) and fibrin Decidual [ce]ll [in] upper part × 15 000

1) Fibrin or derivatives of fibrin
2) Fibrinogen
3) Plasma proteins other than fibrinogen
4) Secretion products

The present findings indicate that the granular material may at leas partly represent fibrin or derivatives of fibrin By light microscopy th MSB and PTAH stains gave positive reactions (red with MSB an bluish black with PTAH) limited to certain areas of the intercellula deposits of the cytotrophoblastic shell whereas Nitabuch s layer gave positive stain throughout the layer at all gestational ages In the elec tron micrographs fibrils with electron density and cross striatio indicative of fibrin were found in association with the granular depos both in the cytotrophoblastic shell and in Nitabuch s layer but not a many as expected from the extent of positivity with MSB and PTA stains Granular masses with the same appearance are found associate with fibrin fibrils in old thrombi on the chorion plate and villi probabl representing aged fibrin (24 26)

By immunohistochemical technique binding of anti fibrin and anti fibrinogen are observed both in the intercellular deposits of the cyto trophoblastic shell and in Nitabuch s layer although the latter give the most uniform reaction (24) However fibrinogen fibrin and aged fibrin are immunological indistinguishable (24) Therefore the granu lar masses observed in this study may also represent fibrinogen as we as aged fibrin For the separation of these two substances the MS and PTAH stains seem unsuitable because *in vitro* examinations hav shown that fibrinogen gives a positive stain with MSB and PTAH i many instances (25)

Thus the granular component of the intercellular deposits of th cytotrophoblastic shell and of Nitabuch s layer may partly be compose of aged fibrin fibrinogen or both

Early stages of lysed fibrin by leucocytes are shown to have a granu lar appearance (30) However fibrinolysis is most likely a factor o insignificant importance since the deposits do not seem to attract man leucocytes and tissue activators of plasminogen are absent in huma placenta (1)

Plasma proteins other than fibrinogen may be components of th granular material because immunohistochemical technique has show the presence of albumin in the intercellular deposit of the cytotropho blastic shell whereas the deposition of this protein in Nitabuch s laye is insignificant (24) The staining reactions with MSB and PTAH o the electron microscopical findings do not give further evidence bu they do not invalidate this theory either The granular material in th cytotrophoblastic shell may therefore partly consist of plasma protein other than fibrinogen whereas that in Nitabuch s layer does not

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Plasma proteins other than fibrinogen may be components of the granular material because immunohistochemical technique has shown the presence of albumin in the intercellular deposit of the cytotrophoblastic shell whereas the deposition of this protein in Nitabuch s layer is insignificant (24) The staining reactions with MSB and PTAH or the electron microscopical findings give further evidence but they do not invalidate this theory either The granular material in the cytotrophoblastic shell may therefore partly consist of plasma proteins other than fibrinogen whereas that in Nitabuch layer does not

In the cytotrophoblastic shell the cells had a well developed Golgi

complex with numerous adjacent smooth surfaced profiles of vacuoles. This is a picture similar to that of other cells possessing secretory activity (11, 13, 28, 34). Previously, it has been assumed on the basis of the glucoprotein content that these cells have secretory activity (7, 37). Ultrastructurally the content of the vacuoles surrounding the Golgi complex was granular as the intercellular material. This is in agreement with *Wislocki & Bennett* (38) who suggested that the intercellular masses are in part secreted by the cytotrophoblastic cells but the evidence is not conclusive.

Thus the intercellular deposits of the cytotrophoblastic shell and Nitabuch's layer contain a material which may represent fibrinogen, fibrin and aged fibrin. In the cytotrophoblastic shell the material additionally may represent other plasma proteins or secretory products of the cytotrophoblasts or both.

Nitabuch's layer probably originates from maternal plasma proteins mainly leaking out from the intervillous space. The great variation in the thickness of the layer may be due to local differences in the permeability of the trophoblastic shell to the penetration of the plasma proteins. At the border between foetal and maternal tissue fibrinogen is in part converted to fibrin. It may also be deposited as fibrinogen since fibrinogen is the most easily precipitated plasma protein (10). A decidual route of the proteins seems more unlikely since no fibrin could be demonstrated between decidual cells in this study. Immunohistochemically only insignificant amounts of plasma proteins are found in the decidual interstitial space (24). Thus the decidua also seems to offer a resistance to further leakage of plasma proteins.

The functional significance of Nitabuch's layer has been the object of several theories. Thus it has been proposed to be an immunological barrier (3, 4). In the human placenta this theory must be questioned because γ globulins are not present (24). Although a granular material can be found in the junctional zone in several species (2, 4, 18, 32, 41) and *Bradbury et al* (4) found that the granular material was more evident in the placentas from hybrid than from inbred mice, other species show a foeto-maternal apposition without significant deposition of granular material (12, 43). Further, no granular material is found in mouse ova transplanted under the kidney capsule of male recipients (33).

Ludwig (22) proposed that Nitabuch's layer offers a protection against the invading trophoblast. Although scattered trophoblastic elements were occasionally found in the decidua, no massive penetration of the trophoblast into the decidua was found in the present study in areas where Nitabuch's layer was absent.

SUMMARY

The basal plate of the normal human placenta is composed of cyto-trophoblastic and decidual cells which may be differentiated both b[y] light and electron microscopy

Rohr s stria bordering the intervillous space is found to be of throm-bogenic origin

The intercellular material of the cytotrophoblastic shell is compose[d] of cellular debris fibrin and a material which ultrastructurally [is] granular to filamentous and is of moderate to low electron density Th[e] granular masses may partly be plasma proteins including fibrinogen leaking from the intervillous space partly aged fibrin and partly [a] secretory product of the cytotrophoblasts

At the junctional zone between cytotrophoblastic and decidual cell[s] there is ultrastructurally no intimate contact between unaltered cyto-trophoblastic and decidual elements They are separated by a laye[r] (Nitabuch s layer) of fibrin cellular debris and granular masses Th[e] granular masses may here represent precipitated fibrinogen or aged fibrin or both

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Key to the abbreviations of figures

C = cytotrophoblast CS = cytotrophoblastic shell D = desmosome DE = decidua F = fibrin G = Golgi complex GR = granular masses IC = intercellular space IV = intervillous space LI = lipid L = lysosome like bodies M = mitochondria N = nucleus NI = Nitabuch s layer P = platelets R = Rohr s stria.

Acta path microbiol scandinav 75 18-26 1969

The Second and Third Departments of Pathology University of Helsinki
Helsinki Finland

MALIGNANT TESTICULAR TUMOURS IN FINLAND

By

LYLY TEPPO

Received 21 x 68

Although the histogenesis of testicular tumours has long been the object of study no generally accepted theory has so far been produced The theory of a germ cell origin which has adherents particularly in U S A (*Friedman & Moore* 1946 *Dixon & Moore* 1952 *Melicow* 1955 1965) is strongly substantiated but the British Testicular Tumour Panel for instance regards the origin of teratoma as an open question (*Collins & Pugh* 1964) The problem has not been solved by chromosome studies either although great hope was placed on this approach (*Galton et al* 1966)

The majority of clinical investigations have been performed on series collected from one hospital (*Culp et al* 1963 *Vechinski et al* 1965)—usually a clinic for radiation therapy (*e g* *Ahlbom* 1947 *Melicow* 1955 *Notter* 1956 *Høst & Stokke* 1959 *Muller* 1962 *Ellman et al* 1965 *Kuttig & Sunaric* 1965)— or on army series (*e g* *Friedman & Moore* 1946 *Dixon & Moore* 1952 1953 *Patton & Mallis* 1959 *Kurohara et al* 1967) Under such circumstances it is not possible to avoid a bias in respect to both the age distribution the frequency of histological types and the choice of treatment given The pathoanatomical studies comprising all testicular tumours from a certain geographical area *e g* a country are few in number (*Collins & Pugh* 1964) Hospital series are of course well suited for the comparison of methods of treatment but they do not offer a reliable basis for the estimation of incidence or frequency of the various histological types in the general population

The low incidence of testicular tumours in Finland has previously attracted attention (*Brotherus* 1959 *Magnus* 1964 *Clemmesen* 1968 but estimates have been based on the data obtained from the Finnish Cancer Registry without a re-evaluation of the histological specimens The purpose of the present investigation was to study the incidence of malignant testicular tumours in Finland and the distribution by age and histological type and to evaluate the results of treatment

Address II Department of Pathology University of Helsinki Haartmaninkatu 3 Helsinki 25 Finland

MATERIAL AND METHODS

The estimation of incidence was based on the new cases of testicular tumour reported to the Finnish Cancer Registry during the years 1953–1961 (Cancer Incidence in Finland 1953–1961). The total number was 193. The actual series studied consists of 131 cases in which sufficient paraffin embedded tissue for histological re-evaluation was obtained. Only malignant tumours are included, Leydig cell tumours for instance being thus omitted from the series. In 26 cases no biopsy had been made and in 36 cases the biopsy specimens of the primary tumour were for one reason or another not available.

The cases omitted being distributed over different laboratories and hospitals, the actual series of 131 cases may be regarded as representative of the situation in Finland at the time in question. One possible source of error seems to be constituted by histologically unconfirmed cases in which the disease mostly was at the terminal clinical stage when it was discovered. These cases represent only some 13 per cent however and cannot significantly influence the distribution by histopathological groups. In the estimation of incidence the are included as appears from the foregoing.

TABLE 1

Distribution of the Series by Histopathological Type

	Histopathological type	Number of cases
I	Seminoma pure	63
II	Embryonal carcinoma pure or with seminoma	26
III	Teratoma pure or with seminoma	[illegible]
IV	Teratoma with either embryonal carcinoma or choriocarcinoma or both and with or without seminoma	[illegible]
V	Choriocarcinoma pure or with either seminoma or embryonal carcinoma or both	4
VI	Non germinal tumours	11
	Total	131

The histopathological classification used was that introduced by American authors (Friedman & Moore 1946, Dixon & Moore 1952, see Table 1 and Fig 1): I seminoma, II embryonal carcinoma, III (adult) teratoma, IV teratocarcinoma, V choriocarcinoma and VI non germinal tumours (sarcoma, lymphoma). Combined tumours were classified according to the more malignant component (Dixon & Moore 1952). On classifying the specimens after re-evaluation the primary diagnoses were disregarded. One to six blocks were available in the various cases. The time interval between this study and the time when the cases were reported to the Cancer Registry ranged from 5 to 13 years, the average being 8 years. In every case it was checked whether the patient was alive or not at the time of this study. In cases where the patient had died the cause of death (according to death certificates) and the survival time after establishment of the diagnosis were obtained from the data of the Cancer Registry. The possible presence of metastases at the time of this survey in patients alive could not be established.

RESULTS

Incidence. The average annual number of testicular tumours reported to the Finnish Cancer Registry was 21, the range being 12 to 28. This makes a mean incidence [illegible] 1 0 new cases per 1 [illegible] 000 men per year. There was no clear tendency towards increase or decrease in the annual number of new cases.

Histopathological [illegible]. The distribution of the cases by histopatho

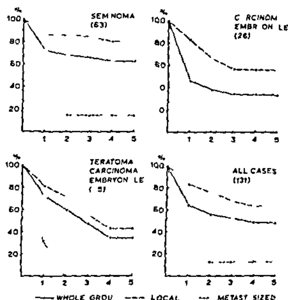


Fig 3

Follow up curves for the whole series and groups I II and IV

64 per cent in the whole group Among 13 patients with metastases two were alive after five years

In the other groups prognosis is poorer In cases of embryonal carcinoma and teratocarcinoma (groups II and IV) the 5 year survival rate is about the same i e 35 and 36 per cent respectively but the 2 year survival rates differ definitely being 39 per cent in cases of embryonal carcinoma and 60 per cent in cases of teratocarcinoma When localized tumours are considered separately the 2 year survival rate is 67 per cent in cases of embryonal carcinoma and 71 per cent in cases of teratocarcinoma and the corresponding 5 year survival rates are 58 and 43 per cent All those patients in group IV (teratocarcinoma) who had metastases at the institution of treatment died within two years of diagnosis those in group II (embryonal carcinoma) within six months

Group III teratoma without malignant components comprised only two patients both of whom had metastases One died three months after the disease had been diagnosed while the other patient was still alive after eight years and a half Four patients with choriocarcinoma died all within six months from the diagnosis Among the 11 patients in the non germinal tumour group five were alive after two years four after five years

The 5 year survival rate for the total series is 48 per cent As to those patients with testicular tumours who survive for five years the prognosis seems to be favourable their expectation of life being about the same as that in a normal population Only one patient in this series ca

case of localized seminoma) has died of his tumour more than five years after the diagnosis was established. The prognosis is more favourable in cases with localized tumours, their 5-year survival rate being 63 per cent in the total series. The prognosis is not quite hopeless even for patients with metastasizing testicular tumours since 13 per cent were alive after five years. All deaths in the group with clinical evidence of metastases at the time of diagnosis occurred within two years from diagnosis.

Therapy. The most common treatment was orchiectomy combined with postoperative radiation (72 per cent). *Surgical treatment alone* was given in 24 per cent. As regards the remaining 6 cases, no data are available. Since the therapy apparently depended on the stage of the disease, a comparison of the methods of treatment cannot be made on the basis of this series.

DISCUSSION

It may be assumed that the data concerning the occurrence of malignant tumours of the testis are reliable considering that the clinical diagnosis of testicular tumours as a rule offers no problem and that the vast majority are malignant and therefore are recorded, e.g. by cancer registries. Hence, series collected from cancer registries are well suited for estimation of incidence. The Finnish Cancer Registry is run on the basis of reports from doctors, hospitals, pathological laboratories and official registries (death certificates). In addition, the Finnish Cancer Registry distributes questionnaires in order to obtain complementary information. In respect to reliability it is on a par with the cancer registries of other countries, only some 5 per cent of all cases being registered on the basis of death certificates only.

The mean annual incidence of testicular tumours diagnosed in Finland during the years 1953–1961 is 1.0 per 100 000 men. This figure is very low as compared with reports from other countries. In Norway, a country resembling Finland in respect to living conditions and population, an annual incidence of 3 new cases per 100 000 men has been reported (*Høst & Stokke* 1959), in Sweden 2.5 new cases per 100 000 men (Cancer Incidence in Sweden 1959). In various reports from U.S.A. and Great Britain the corresponding figure has been 2–3 (*Dixon & Moore* 1952, *Collins & Pugh* 1964). As the low Finnish estimate can hardly be due to errors of diagnosis or deficient records, it is apparent than an actual difference is involved.

The distribution of testicular tumours by histopathological groups varies widely in different series. The frequency of seminoma was 34 per cent in *Dixon & Moore's* series from U.S.A., 40 per cent in a series from Great Britain (*Thackray* 1964). In the Scandinavian countries higher figures have been [illegible] 8 per cent (*Nolte* 1955, *Høst & Stokke* 1959, Cancer Incidence in Sweden 1959, *Muller* 1962, *Ekman et al.* 1965). The frequency [illegible] inland 48 per cent is of the

same order as these and the difference as compared with American series is noteworthy

As regards embryonal carcinoma and teratoma (groups II III and IV) a reverse difference is observed In the series reported by *Friedman & Moore* the frequency of teratomatous tumours was 64 per cent The corresponding figure for Finland 40 per cent is closer to the figures indicated in the other Scandinavian countries *i e* 33–40 per cent It must of course be borne in mind that different investigators may have used different criteria of histopathological classification On the other hand it may be assumed that for instance seminoma is classified as such everywhere in the world owing to its typical histopathological picture And if all teratomatous tumours are united to form one group the pertinent figures are comparable as well In Finland a clear trend is observed Seminomas are common teratomatous tumours are infrequent This is in agreement with the situation in the other Scandinavian countries The impression that teratomatous tumours are rare in Finland as compared with other countries is strengthened when the low total incidence of testicular tumours is related to the high percentage of seminoma For final assessment of this question further studies are required however

The age distribution both in the whole series and in the different histopathological groups corresponds to that established in previous investigations The median age of the patients with seminoma was about 40 years *i e* about 10 years more than the median age of the patients with teratomatous tumours Sarcoma was most frequently encountered in elderly men as is usually the case with somatic tumours Neither seminoma nor teratoma or teratocarcinoma was detected in children No pubertal cases occurred which is in agreement with many previous reports

Both in the whole series of testicular tumours which represents Finland as a geographical entity and in the different histopathological groups the prognosis was in agreement with findings reported in previous studies (*Dixon & Moore* 1952 *Pugh & Cameron* 1964) Seminoma was found to have the most favourable prognosis the 5 year survival rate in cases with localized tumours being 81 per cent The figures indicated from other countries have as a rule ranged from 80 to 90 per cent The prognosis in cases of embryonal carcinoma was more favourable in the present series than in a report from U S A As to localized tumours the 5 year survival rate was 58 per cent in this series while *Dixon & Moore* indicated 35 5 per cent As to teratocarcinoma the corresponding figures were 43 per cent (the present series) and 47 8 per cent (U S A) The combined 4 year survival rate in cases of localized "malignant teratoma anaplastic intermediate B and intermediate A corresponding to embryonal carcinoma and teratoma in the nomenclature used in this study was estimated at 43 per cent in Great Britain (*Pugh & Cameron* 1964)

SUMMARY AND CONCLUSIONS

During the years 1953–1961 the total number of new cases of testicular tumours reported to the Finnish Cancer Registry was 193 In 131 cases paraffin embedded tissue was obtained for re evaluation The distribution of testicular tumours by age and histological type and the results of treatment were studied on the basis of this series

The following conclusions were drawn

1) The incidence of testicular tumours is low in Finland *i e* 1 0 per 100 000 men per year which is less than figures reported from other countries
2) Of the different histopathological types seminoma is relatively more common in Finland (48 per cent) than in U S A and Great Britain for instance but of the same order of frequency as in the other Scandinavian countries Teratomatous testicular tumours are rare in Finland
3) Both in the whole series and in different histopathological groups the age distribution is in agreement with previous reports from other countries
4) The prognosis is roughly the same as is other countries

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Acta path microbiol scandinav 75 27 32 1969

The Division of Biological and Medical Research Argonne National Laboratory Argonne Illinois and Pediatric Research Laboratory Variety Club Heart Hospital University of Minnesota Minneapolis Minnesota and the Department of Histology Institute of Human Anatomy University of Uppsala Uppsala, Sweden

RADIOSENSITIVITY OF THE LYMPHOCYTES WITHIN THE GUT EPITHELIUM

By

K E Fichtelius

Received 14 v 68

As long as lymphocytes have been recognized they have been observed in large numbers in the gut epithelium (15) Most of the lymphocytes within the gut epithelium here called theliolymphocytes are situated between the epithelial cells close to the basement membrane (1 3 6 10 12) During the years the theliolymphocytes have been studied there has been a debate whether they simply migrate through the epithelium and are lost in the lumen of the gut or whether they perform some kind of function (4 15)

Two recent publications have revived this discussion and brought the theliolymphocytes into focus The kinetics of the theliolymphocytes in rat has been studied by Fichtelius with the aid of H^3 thymidine labelling (6) It is evident from this study that at least 2–3 per cent of the theliolymphocytes synthetize DNA *in situ* As many as 12 per cent of the theliolymphocytes at the tip of the villi of the small intestine are less than three days old These data can be best explained by the assumption that the theliolymphocytes are to a large extent immigrants from the blood and that these immigrants constitute a selection of young lymphocytes compared to the ordinary mixture of mostly old blood lymphocytes There is some indication from this study that the theliolymphocytes do re enter lamina propria that they are not all shed off into the lumen together with the epithelial cells

Meader & Landers (12) in a recent study of the ultrastructure of the theliolymphocytes demonstrate that at least some theliolymphocytes do re enter lamina propria This conclusion was based on the direction of the collagen fibres at the rupture of the basement membrane following the passage of a lymphocyte and on the location of the cytoplasmatic tail of the moving lymphocytes in relation to the nucleus

Visiting Resear[illegible] f[illegible] the Am[illegible] Heart [illegible]
The author is very [illegible] d to Dr Sa[illegible]uel Lesher [illegible] A[illegible] ne National Laboratory who [illegible] ated [illegible] giv e me access to the material or [illegible]

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RADIOSENSITIVITY OF THE LYMPHOCYTES WITHIN THE GUT EPITHELIUM

By

[illegible] FICHTELIUS

Received [illegible]

As long as lymphocytes h[illegible] en recognized they have been observed in large numbers in the [illegible] thelium (15) Most of the lymphocytes within the gut epitheliu[illegible] called theliolymphocytes are situated between the epithelial [illegible] se to the basement membrane (1 3 6 10 12) During the [illegible] theliolymphocytes have been studied there has been a deb[illegible] whether they simply migrate through the epithelium and are lost [illegible] n of the gut or whether they perform some kind of functio[illegible]

Two recent public[illegible] revived this discussion and brought the theliolymphocytes [illegible] The kinetics of the theliolymphocytes in rat has been studi[illegible] thus with the aid of H³ thymidine labelling (6) It is evid[illegible] this study that at least 2–3 per cent of the theliolymphocyt[illegible] synthetize DNA *in situ* As many as 12 per cent of the theliolymp[illegible] cytes at the tip of the villi of the small intestine are less than [illegible] old These data can be best explained by the assumption th[illegible] iolymphocytes are to a large extent immigrants from the blo[illegible] that these immigrants constitute a selection of young lymp[illegible] compared to the ordinary mixture of mostly old blood lympho[illegible] There is some indication from this study that the theliolymphocytes do re[illegible] nt r lamina propria that they are not all shed off into the lum[illegible] together with the epithelial cells

Meader & Landers (12) in a recent study of the ultrastructure of the theliolymphocytes demonstrate that at least some theliolymphocytes do re enter lamina propria This conclusion was based on the direction of the collagen fibres at the rupture of the basement membrane following the passage of a lymphocyt[illegible] and on the location of the cytoplasmic tail of the moving lymphocytes in relation to the nucleus

Visiting Research Profe[illegible] or [illegible] f the Am[illegible] an Heart A sociatio[illegible]

The author is very much in[illegible] Dr S[illegible]nuel Lesher at the A[illegible]onne National Laboratory who generousl[illegible] c[illegible] l[illegible] rated thr[illegible]gh ut this stu[illegible] and gave [illegible] the material on which it is ba[illegible]

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Acta path microbiol scandinav 7 ?–32 1969

The Division of Biological and Medical Research Argonne National Laboratory Argonne Illinois and Pediatric Research Laboratory Variety Club Heart Hospital University of Minnesota Minneapolis Minnesota and the Department of Histology Institute of Human Anatomy University of Uppsala Uppsala Sweden

RADIOSENSITIVITY OF THE LYMPHOCYTES WITHIN THE GUT EPITHELIUM

By

K. I. ... TELIUS

Received 14 v 68

As long as lymphocytes have been ... in large numbers in the gut epith... within the gut epithelium here ... between the epithelial cells close ... (10 1?) During the years the ... there has been a debate whether ... thelium and are lost in the lumen ... some kind of function (4 15)

Two recent publications have ... the theliolymphocytes into focus ... in rat has been studied by Fichtel... labelling (6) It is evident from th... of the theliolymphocytes synthetize ... cent of the theliolymphocytes at th... testine are less than three days old ... by the assumption that the thelioly... immigrants from the blood and th... selection of young lymphocytes com... mostly old blood lymphocytes Ther... that the theliolymphocytes do re ent... not all shed off into the lumen toget... *Meader & Landers* (12) in a recent ... theliolymphocytes demonstrate that ... re-enter lamina propria This concl... of the collagen fibres at the rupture of ... ing the passage of a lymphocyte and ... material of the moving lymphocytes ...

... bers have ... observed ... Most of the ... hocytes ... cytes ... ated ... ent membran... ... been ... ... through ... whether they ...

... liseus ... and ... the theliolymph... ... of ... ... at least ... 3 per c... ... As many ... 12 p... f the villi of the ... all in ... data ... ... defined ... cytes are ... large extent ... immigrants ... tute a ... to the ordinary ... ure of ... ... from ... tudy ... that ... are ... epithelial cell ...

... ... f the ... theliolymphocytes ... ... direction ... ... allow ... ... ext plas... ... ...

... Research Professor of the Ameri... ... one ... ional
The author is very much indebted to Dr. S... ... ... have ... access
... who generously collaborated throu...
... the material on which it is based.

Before these studies were made Fichtelius (5) regarding it unlikely that all the theliolymphocytes are destined to be lost into the intestinal lumen advanced a theory that the epithelium of he entire gut in some animals such is fish is a first level lymphoid organ According to this theory the gut epithelium exerts an influence on lymphocytes and lymphoid organs similar to that of bursa Fabricii in birds This theory is discussed elsewhere in the light of new findings about the phylogeny of lympho epithelial relationships (9) and in the relation to the phenomenon of homing of lymphocytes to the gut epithelium (7 8)

The present study deals with the radiosensitivity of the theliolymphocytes The radiation damage can be either a reproductive death of cells or an *interphase death of cells (14) In reproductive* death after irradiation the cells continue to survive for a considerable length of time They may divide several times or they may form giant cells before they finally die In the case of interphase death on the other hand the cells within a matter of a few hours undergo degenerative changes culminating in their disappearance from the tissues It is the rapid disappearance of the theliolymphocytes and their reappearance which is examined here

MATERIAL AND METHODS

Male and female 100 day old BCF_1 mice (C57Bl♀ × BALB♂) were exposed to a 300 R whole body ^{60}Co gamma ray dose The effects of 300 R on cell proliferation has been reported in a recent paper (11) Mice were separated two to a plastic cylindrical cage (5 × 6 inches) at time of exposure Cages were positioned in an irradiation chamber equipped with a 2000 Ci ^{60}Co source The 300 R (air) dose was delivered in a 15 minute interval starting at 0815 (dose rate 20 R/min) The mid line tissue dose was 273 rads

Thirteen animals were killed 6 hours after irradiation and 12 animals were killed 12 hours later *Seven or eight animals were killed on each of the following times* after irradiation 24 48 and 96 hours and 8 days Half of the animals in each group were labelled with H^3 thymidine 30 or 60 minutes before they were killed The other half of the animals were labelled in the same way 15–16 hours before they were killed All animals received 10 uC of H^3 thymidine intraperitoneally (from Schwartz Bio Research Inc 0 36 C/mM)

Autoradiographs were prepared from fixed embedded sectioned (3 microns) pieces of duodenum All sections were Feulgen stained

Two thousand epithelial nuclei were counted in sections from every animal and the number of theliolymphocytes among these 2 000 epithelial nuclei was recorded Only perpendicularly sectioned epithelium of the villi was examined and in every instance the uttermost tips of the villi were avoided

The percentage of labelled theliolymphocytes were counted in the same position in the epithelium Every theliolymphocyte with 5 grains or more above the nucleus was regarded as labelled Four hundred theliolymphocytes were examined in every slide All counting work was done by a specially trained technician who was un*aware of the design of the experiment* With the Feulgen stained used the basement membrane is often impossible to see and some lymphocytes situated in lamina propria may have been falsely counted as theliolymphocytes

RESULTS

The number of theliolymphocytes The normal number of theliolymphocytes per 2000 epithelial nuclei seems to be a little less than 200

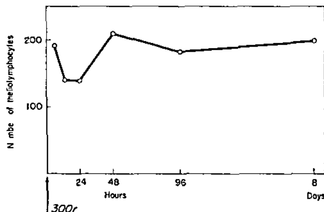


Fig 1

The number of theliolymphocytes per 2000 epithelial nuclei at different times after irradiation

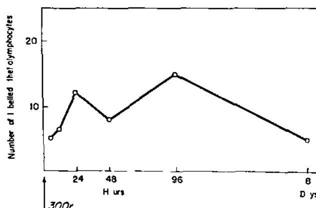


Fig 2

The number of labelled theliolymphocytes per 400 theliolymphocytes in mice labelled ½–1 hour before killing

or close to 10 per cent in this Feulgen stained material and with this particular technician counting See Fig 1 In the animals killed 12 and 24 hours after irradiation the number of theliolymphocytes were decreased the means being 140 and 139 respectively per 2000 epithelial nuclei These values differed at an 0 01 level from the values in animals killed later (Mann Whitney U test)

The number of labelled theliolymphocytes 30 minutes—1 hour after injection of H³ thymidine The number of labelled theliolymphocytes per 400 theliolymphocytes in this series showed great variation and no certain pattern can be seen See Fig 2 It cannot of course be excluded

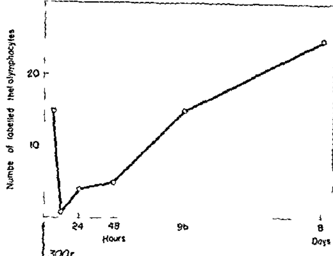


Fig 3

The number of labelled theliolymphocytes per 400 theliolymphocytes in mice labelled 15 16 hours before killing

from this small series that radiation has an influence on this particular type of theliolymphocytes synthesizing DNA *in situ*

The number of labelled theliolymphocytes 15–16 hours after injection of H^3 thymidine The number of labelled theliolymphocytes per 400 in this series showed a clear cut pattern after irradiation There was a minimum of labelled theliolymphocytes 12 24 and 48 hours after irradiation around 3 labelled cells per 400 See Fig 3 The highest figure 24 9 on day 8 after irradiation is statistically higher than these lower values taken together at the 0 01 level (Mann Whitney U test)

DISCUSSION

The number of theliolymphocytes decreased at about the same time as blood lymphocytes have been shown to decrease in similar experiments (2) The number of theliolymphocytes is however restored to normal values much faster than is the number of blood lymphocytes in comparable experiments (2 13) The transit time of lymphocytes within the gut epithelium cannot be more than 2 3 days (6) A plausible explanation of the rapid reappearance of theliolymphocytes is therefore that the main source of theliolymphocytes is not damaged by irradiation but the lymphocytes within the epithelium are An alternative but less likely explanation is that the source of theliolymphocytes is restored with extreme speed

The mean percentage of labelled theliolymphocytes 30 minutes to 1 hour after labelling was 1 9 This figure is in agreement with earlier data for mice (3) and for rats (6) The higher figure 15 16 hours after labelling in the 8 day animals [illegible] per cent is also in agreement

with earlier findings (6) As in rats this increase with time of labelled theliolymphocytes after a single injection of H³ thymidine is probably due to a selective immigration of young lymphocytes from the blood

In the animals labelled 15–16 hours before killing there was a clear cut effect of irradiation on the number of labelled theliolymphocytes the figures being very low 12 24 and 48 hours after irradiation In this part of the experiment the majority of the labelled theliolymphocytes were labelled before they entered the epithelium and became theliolymphocytes The 12 hour animals were irradiated 4 hours after labelling the 24 hour animals were irradiated 8 hours before labelling and the 48 hour animals were irradiated 32 hours before labelling This means that irradiation between 32 hours before until 4 hours after labelling did hit part of the presumptive theliolymphocytes in their source namely those in DNA synthesis

It cannot of course be excluded from this small series that also the *theliolymphocytes synthetizing DNA in situ are killed by 300 r*

The source of theliolymphocytes is unknown so far For theoretical reasons the bone marrow is the most likely source of these cells in adult mammals (8) According to this study the largest part of the source could be radioresistant (or extremely rapidly restorable) The DNA synthetizing part of the source seems to be radiosensitive The bone marrow could certainly include a population of presumptive theliolymphocytes fitting these criteria

SUMMARY

The lymphocytes of the gut epithelium of mammals may prove to be very interesting from a biological point of view The whole gut epithelium may function as a first level lymphoid organ It has been shown *in rats that there is an immigration of a selection of young lymphocytes* from the blood Study of the ultrastructure of the gut epithelium has revealed that lymphocytes of the epithelium re enter lamina propria The present study deals with the radiosensitivity of these lymphocytes (here called theliolymphocytes) in mice given 300 R and labelled with H³ thymidine at different times

It is shown that the theliolymphocytes on the whole are radiosensitive in the same way as blood lymphocytes The main part of the unknown source of theliolymphocytes seems to be radioresistant The DNA synthetizing part of this source seems to be radiosensitive

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Acta path. microbiol scandinav 75 33-36 1969

The University Institute for Experimental Medicine
Nørre Alle 71 2100 Ø Copenhagen Denmark

REINVESTIGATION OF THE CAUSE OF THE INCREASED VELOCITY OF REACTION BETWEEN RENIN AND ANGIOTENSINOGEN IN PLASMA FROM NEPHRECTOMISED RATS

By

JENS BING

Received 14 vi 68

The velocity of the reaction between renin and its substrate angiotensinogen and the subsequent formation of angiotensin is known to be higher in plasma from nephrectomised animals than in plasma from normal animals This difference has been believed to be caused by the nephrectomy induced increase in the concentration of angiotensinogen in plasma because the increase in angiotensin yield parallels the increase in angiotensinogen concentration (*Bing* 1964) In accordance with this *Blaquier et al* (1967) found no difference in the Michaelis constant when plasmas from normal or 24 hour nephrectomised rats were used Other investigators have however published reports presenting evidence that the change of renin reactivity following nephrectomy is due to loss of a humoral factor of renal origin probably a renin inhibitor in nephrectomised animals (*Brunner* (1962) *Hoobler et al* (1964) *Sen et al* (1967 and 1968) *Smeby et al* (1967)) It was therefore thought desirable to reinvestigate the problem

METHODS

The *concentration of angiotensinogen* in heparinized plasma was determined by incubation for 20 at 37 C of 50 µl plasma or diluted plasma with 250 µl of a solution of partly purified renin containing 0 7 Haas Goldblatt standard renin units per ml The renin was the commercial preparation of N B C Cleveland Ohio which was tested against the standard donated by Dr *Haas* to the W H O Lab for Biological Standards (Nat Inst Med Res Mill Hill London) The unit of the commercial preparation was found to be about one third of that of the standard In this standard techniqu renin was present in excess

Before the incubation partial ina ti ation f angiotensinases was carried out by acidif cation of plasma with 2 N phosphoric acid to pH 3 C for 20 min at 25 C followed by readjustment of the pH to 7 5 with 6 N NaOH

After the incubation during wh ch ti ne the mixture was covered with 1 2 ml of

Supported by grants from *K ng Ch   t   X s Foun lation* and the *grant of Ebba Celinder* The author is indebt d t *D F Haa* Cleveland for gene ous supply of standard renin

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Acta path microbiol scandinav 75 37-50 1969

Ullevål Hospital Department of Pathology University of Oslo Oslo Norway

THE ADRENAL WEIGHT OF MICE WITH SPONTANEOUS ADRENOCORTICAL LIPID DEPLETION

A Comparison with Untreated ACTH and Dexamethasone Treated C57Bl Mice

By

KARE MOLNE

Received 25 x 68

The spontaneous adrenocortical lipid depletion was originally observed in the AKR/O strain of mice which has a high incidence of spontaneous lymphatic leukaemia (1 2) From hybridization experiments carried out between the AKR/O strain and a normal strain WLO the two different lines CS and AC have emerged Both substrains have the same specific depletion of adrenocortical lipids but in contrast to the CS strain the AC strain has been cleared from susceptibility to leukaemia (3)

The spontaneous adrenocortical lipid depletion depends upon one recessive gene (3) In terms of Sudanophilia the lipid depletion is almost total in adult males In females the depletion of lipids is subtotal with some variation from animal to animal (2)

The adrenocortical lipid depletion is not present at birth It is manifest only in sexually mature animals Studies on male mice of the AC substrain have demonstrated that the reduction of Sudanophilia in the adrenal cortex coincides with the degeneration of the adrenal X zone and with the maturation of the seminiferous epithelium (13) The appearance of lipid depletion can be prevented by prepubertal gonadectomy (2)

Cortisone treatment (4) and hypophysectomy experiments (7) have demonstrated that the specific adrenocortical lipid pattern of these animals is related to the function of the pituitary gland After hypophysectomy a rapid reaccumulation of lipids takes place in the adrenal cortex This lipid repletion can be partly prevented by large doses of ACTH in the postoperative period

Ultrastructurally there is a characteristic difference between normal

I am indebted to the head of the Life Insurance Companies Institute for Medical Statistics at the Oslo City Hospitals dr *Knut Westlund* for his helpful advice regarding the statistical analyses.

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[illegible] Department of [illegible] University of [illegible]

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By

KARE MOLNE

Received 2[illegible] 63

The spontaneous adrenocortical lipid depletion was originally observed in the AKR/O strain of mice which has a high incidence of spontaneous lymphatic leukaemia (1 2). From hybridization experiments carried out between the AKR/O strain and a normal strain WLO the two different lines CS and AC have emerged. Both substrains have the same specific depletion of adrenocortical lipids but in contrast to the CS strain the AC strain has been cleared from susceptibility to leukaemia (3).

The spontaneous adrenocortical lipid depletion depends upon one recessive gene (3). In terms of Sudanophilia the lipid depletion is almost total in adult males. In females the depletion of lipids is subtotal with some variation from animal to animal (2).

The adrenocortical lipid depletion is not present at birth. It is manifest only in sexually mature animals. Studies on male mice of the AC substrain have demonstrated that the reduction of Sudanophilia in the adrenal cortex coincides with the degeneration of the adrenal X zone and with the maturation of the seminiferous epithelium (13). The appearance of lipid depletion can be prevented by prepubertal gonadectomy (2).

Cortisone treatment (4) and hypophysectomy experiments ([illegible]) have demonstrated that the specific adrenocortical lipid pattern of these animals is related to the function of the pituitary gland. After [illegible]physectomy a rapid reaccumulation of lipids takes place in the ad[illegible] cortex. This lipid repletion can be partly prevented by large d[illegible] ACTH in the postoperative period.

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age the animals are sexually mature and in the AC strain the spontaneous adreno cortical lipid depletion i complete I (13) The following series are included

A One hundred AC and one hundred C57Bl male mice given no hormone treatment collected from October 1965 to February 1966 The series comprises all males born during the period of investigation except 90 males from each strain which were used for breeding

B Fifty six AC and sixty C57Bl male mice given no hormone treatment collected from September 1966 to March 1967 Series B consists of all males born in the period referred to except 25 animals from each strain which were put up for breeding and the animals which were used in the hormone experiments described below

C Thirty AC and thirty C57Bl male mice collected from September 1966 to January 1967 and given ACTH for 3 weeks as described below

D Thirty two AC and thirty C57Bl male mice collected from September 1966 to January 1967 and given dexamethasone for 3 weeks as described below

E Twenty AC and thirty three C57Bl male mice given no treatment collected from September 1966 to January 1967 These animals serve as controls for the hormone experiments and represent the first part of series B The rest of series B also comprises animals collected *after* the hormone experiments were ended (January to March 1967)

Procedure for the Hormone Experiments (Series C D F)

In series C D and I body weight was recorded at the start of the hormone experiments which lasted for 3 weeks The animals of these series were taken by random Within each strain the body weight of mice in the different groups was found to be of the same range In each cage all animals got an identical treatment All injections were performed between 8 and 10 A M and were made subcutaneously in the intrascapular region

Pilot studies on C57Bl male mice showed that within certain limits increasing effect upon adrenal weight could be achieved with increasing doses of the hormones For the comparative studies doses were chosen which lead to a significant alteration of adrenal weight but not to the maximal effect

In series C the ACTH preparation Jaton prolongatum A L was used This preparation is purified from pig pituitary glands and is made for depot effect From the age of 57 59 days 0 03 ml (1 8 i u of ACTH) was given daily The animals were killed 1 hour after the last injection

In series D 0 025 ml of Decadron M S D (0 1 mg of dexamethasone) was given every second day from the age of 57 59 days Dexamethasone has a potent glucocorticoid [?] effect and was used as depressor of endogenous pituitary function The animals were killed 2 hours after the last injection

The possible effect of the handling [illegible] animals and of the [illegible] for the hormones was tested Seve[illegible] C57Bl male [illegible] given subcutaneous injections of 0 03 ml of [illegible] vehicle for Jaton [illegible] tum A L[1] daily for 3 [illegible] milarly [illegible] C57Bl male were given 0 025 ml of saline [illegible] usly every second day for three weeks

[1] Provided through [illegible] A S [illegible] Fernes [illegible] rium for Special preparater O [illegible] Chemi[illegible] rat [illegible] Copenhagen

TABLE

Adrenal Weight (mg) Body Weight (g) and Relative Adrenal

Series	Number AC	Number C57Bl	Adrenal weight AC Mean	Adrenal weight AC SD	Adrenal weight C57Bl Mean	Adrenal weight C57Bl SD	P
A Oct 1965–Feb 1966	100	100	2 83	0 32	2 86	0 35	N S
B Sept 1966–March 1967	56	60	3 10	0 27	3 09	0 26	N S.

S D Standard deviation
P Significance level of difference between strains

These doses correspond to those given in the hormone experiments Thirty three untreated animals of the same strain served as controls for these experiments Body weight of and adrenal weight in mice in the treated groups and in the control groups were not found to differ significantly

Weighing procedure The weighings were performed between 8 and 10 A M The animals were weighed on a Cenco balance to the nearest 0 1 g When sacrificed, they were decapitated with scissors and bled The adrenals were dissected out with surrounding fat tissue and put in saline in a Petri dish which was covered with a glass plate to avoid evaporation The adrenals were carefully dissected free from fat under a dissection microscope and weighed on a H 16 Mettler balance (Precision 0 01 mg readibility 0 01 mg) These procedures were carried out by two trained technicians Before weighing the adrenals were quickly dried on the same type of filter paper Thereafter they were placed on a tared watch glass and weighed to the nearest 0 01 mg Tests showed that the weight loss due to evaporation was about 4 per cent 60 seconds after the adrenals had been placed on the weighing pan The whole procedure from the sacrifice of the animals to the weighing of the adrenals was strictly standardized The technicians were the same throughout the study They had no specific knowledge about the problems dealt with

Statistical methods Mean values in the two strains were compared by means of the Student s t-test The relationship between body weight and adrenal weight was examined by means of an analysis of covariance Probabilities under the O hypothesis (P) > 0 05 have been considered not significant (N S)

RESULTS

Untreated AC and C57Bl Male Mice

The means and standard deviations of absolute adrenal weight body weight and relative adrenal weight (mg/100 g body weight) in series

1
Weight (mg/100 g Body Weight) in Male Mice 78–80 Days Old

Body weight					Relative adrenal weight				
AC		C57Bl		P	AC		C57Bl		P
Mean	S D	Mean	S D		Mean	S D	Mean	S D	
21 9	1 6	24 3	2 1	<0 01	13 0	1 5	11 8	1 5	<0 01
23 [illegible]	1 7	25 4	2 1	<0 01	13 4	1 2	1[illegible]	1 2	<0 01

N S No significant difference

A and B are given in Table 1 Figs 2 and 3 are scatter diagrams of results in series A relating adrenal weight to body weight in each strain Regression lines fitted by the method of least squares are drawn and the 95 per cent confidence intervals for the lines are indicated

The accumulation of animals from the two strains during the period of collection for series A is shown in Fig 1 The number of animals from the two strains was roughly equal throughout the period

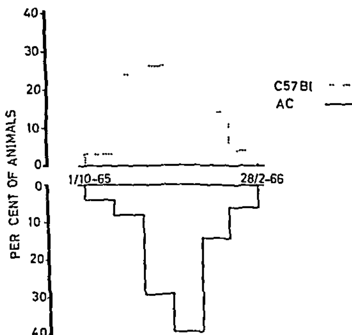


Fig 1

The accumulation of AC and C57Bl animals during the period of collection of series A The series consist of 100 males from each strain

TABLE 3

Relative Adrenal Weight (mg per 100 g Body Weight) in Male Mice Treated from the Age of 57–59 Days to the Age of 78–80 Days with ACTH and Dexamethasone. The Adrenal Weight Has Been Related to the Body Weight both at the Beginning and the End of the Experiments

Series	No of animals AC	No of animals C57Bl	Relative adrenal weight (body weight at 57–59 days) AC Mean	AC SD	C57Bl Mean	C57Bl SD	P	Relative adrenal weight (body weight at 78–80 days) AC Mean	AC SD	C57Bl Mean	C57Bl SD	P
C ACTH treatment[1]	30	30	25.6	2.4	23.8	2.6	<0.01	24.7	2.4	22.7	2.8	<0.01
D Dexamethasone treatment[2]	32	30	12.2	1.4	11.4	1.7	0.05>P>0.01	19.2	1.2	11.4	1.7	0.05>P>0.01
I Controls	20	33	15.4	1.3	14.3	1.5	<0.01	13.7	0.9	12.6	1.4	<0.01

SD *Standard deviation*
P Significance level of difference between strains
1 1.8 i.u. every day 2 0.1 mg every second day

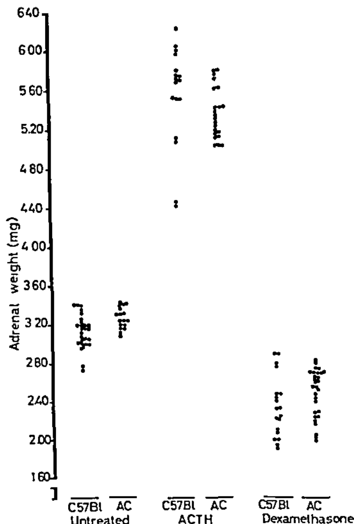


Fig 4

Effect of ACTH and dexamethasone treatment upon absolute adrenal weight in AC and C57Bl mice (see text) Each point represents one animal

AC and C57Bl Male Mice Given Treatment with ACTH or Dexamethasone

The effect of long term treatment with ACTH and dexamethasone upon the adrenal weight of the two strains are shown in Tables 2 and 3 and Figs 4 and 5

The hormone treatment had the same effect upon absolute adrenal weight in both strains Both in the ACTH treated and in the dexametha sone treated groups the mean values for the adrenal weight are almost identical in the two strains (Table 2) and the scatter diagrams (Fig 4)

TABLE 3

Relative Adrenal Weight (mg per 100 g Body Weight) in Male Mice Treated from the Age of 57–59 Days to the Age of 78–80 Days with ACTH and Dexamethasone. The Adrenal Weight Has Been Related to the Body Weight both at the Beginning and the End of the Experiments

Series	No of animals		Relative adrenal weight (body weight at 57–59 days)					Relative adrenal weight (body weight at 78–80 days)				
	AC	C57Bl	AC		C57Bl		P	AC		C57Bl		P
			Mean	SD	Mean	SD		Mean	SD	Mean	SD	
C ACTH treatment[1]	30	30	25.6	2.4	23.8	2.6	<0.01	24.7	2.4	22.7	2.8	<0.01
D Dexamethasone treatment[2]	32	30	12.2	1.4	11.4	1.7	0.05>P>0.01	12.2	1.2	11.4	1.7	0.05>P>0.01
L Controls	20	33	15.4	1.3	14.3	1.5	<0.01	13.7	0.8	12.6	1.4	<0.01

SD Standard deviation
P Significance level of difference between strains
1 1.8 i.u. every day 2 0.1 mg every second day

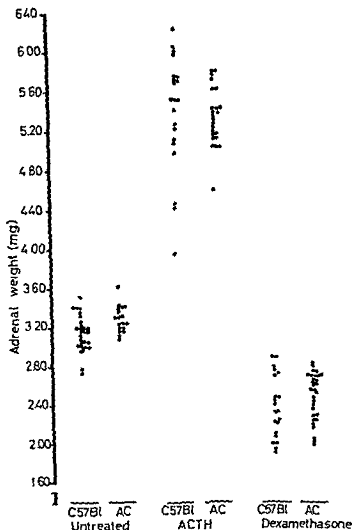


Fig 4

Effect of ACTH and dexamethasone treatment upon absolute adrenal weight in AC and C57Bl mice (see text) Each point represents one animal

AC and C57Bl Male Mice Given Treatment with ACTH or Dexamethasone

The effect of long term treatment with ACTH and dexamethasone upon the adrenal weight of the two strains are shown in Tables 2 and 3 and Figs 4 and 5

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Series	No of animals		Relative adrenal weight (body weight at 57–59 days)					Relative adrenal weight (body weight at 78–80 days)				
	AC	C57Bl	AC		C57Bl		P	AC		C57Bl		P
			Mean	SD	Mean	SD		Mean	SD	Mean	SD	
C ACTH treatment[1]	30	30	25.6	2.4	23.8	2.6	<0.01	24.7	2.4	22.7	2.8	<0.01
D Dexamethasone treatment[2]	32	30	12.2	1.4	11.4	1.7	0.05>P>0.01	12.2	1.2	11.4	1.7	0.05>P>0.01
E Controls	20	33	15.4	1.3	14.3	1.5	<0.01	13.7	0.8	12.6	1.4	<0.01

SD Standard deviation
P Significance level of difference between strains
1 1.8 I.U. every day
2 0.1 mg every second day

two strains Thus as regards adrenal weight both strains react in the same way to adrenocortical stimulation and to inhibition Thus if the spontaneous adrenocortical lipid depletion is caused by an altered pituitary adrenocortical function this feature is not revealed in the weight of the adrenals

SUMMARY

The adrenal weight has been determined in untreated ACTH treated and dexamethasone treated mice with spontaneous adrenocortical lipid depletion and in mice with a normal adrenocortical lipid pattern (C57Bl)

The relative adrenal weight in untreated mice with spontaneous adrenocortical lipid depletion is significantly higher than that in C57Bl mice The absolute adrenal weights in mice in the two strains are practically identical while the body weights are significantly different The alterations in adrenal weight and body weight after long term treatment with ACTH and dexamethasone are very similar in the two strains

The values for relative and absolute adrenal weight in both strains are within the range of those which according to reports have been obtained in the case of normal mice

The findings do not support the hypothesis that there is an abnormal pituitary adrenocortical function in mice with spontaneous adreno cortical lipid depletion

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TABLE 4

Data from 2 Studies on Adrenal Weight in Male Mice
The Results from the Present Study Have Been Included in the Table

Study	Strains of mice	No of animals	Age (days)	Adrenal weight (mg)	Relative adrenal weight (mg/100 g body weight)
Chester Jones 1957	Strong A	45	60–120	2 58	11 4
	Bagg C albino	28	120	3 50	13 3
Thiessen 1964	C57Bl	10	77–80	2 49	10 8
	BALB/c	10	77–80	3 04	11 8
	DBA/2	10	77–80	2 33	11 5
	A	10	77–80	2 19	9 9
	C3H/2	10	77–80	2 89	14 8
	R III	10	77–80	3 18	11 5
Present study series A	AC	100	78–80	2 83	13 0
	C57Bl	100	78–80	2 86	11 8

TABLE 5

Examples of Adrenal Weight in Female Mice with Well Characterized Endocrinopathies

Authors	Endocrinopathy	No of animals	Adrenal weight (mg)	Relative adrenal weight (mg/100 g body weight)
Bahn et al 1957	Implanted ACTH producing tumours	11	16 6	55
	Controls	13	6 4	27
Smith et al 1930	Hereditary dwarfism (pituitary hypofunction)	5	0 3–1 0	8–17
	Controls	2	5 3–7 2	33
all et al [illegible]	Hereditary obese hyper glycaemic syndrome	4	8 8	13
	Controls	4	5 0	17

Calculated by the author on the basis of mean values for adrenal weight and body weight

in mice is to be taken as an indication of pituitary adrenocortical dys function only very marked differences should be considered and abso lute as well as relative values must be taken into account

It must therefore be concluded that in spite of a significantly higher *relative* adrenal weight in untreated AC male mice compared with that in C57Bl male mice this difference is too small (Table 4) to prove an abnormal adrenocortical function in mice with spontaneous adreno cortical lipid depletion

This conclusion is further supported by the ACTH and dexametha sone experiments (Tables 2 and 3 and Figs 4 and 5) The absolute adrenal weight after hormone treatment was not found to differ in the

duction is probably lower in CS mice after ACTH injection than in normal control animals subjected to the same treatment (20) Therefore it is not clear whether the spontaneous adrenocortical lipid depletion reflects an anomaly of pituitary function or a primary anomaly of the adrenal cortex itself

Studies of the organ weights gave no obvious indication of a different functional state of the adrenal cortex in these mice Although the adrenal weight per 100 grams of body weight is significantly higher in AC mice than in one control strain (C57Bl) to which they were compared (15) the relative weight does not differ markedly from findings in other presumably normal strains of mice

Numerous investigations have shown that variations in the pituitary stimulation of the adrenal cortex are reflected in the width of the different cortical zones and in the size of cell nuclei and nucleoli (11 12 13 16 22 23) In the present work these parameters have been used to test whether the permanent adrenocortical lipid depletion is a direct result of an altered pituitary influence upon the adrenal cortex To this end untreated AC males were compared with untreated ACTH treated and dexamethasone treated C57Bl males Dexamethasone is a well known depressor of pituitary ACTH secretion

MATERIAL AND METHODS

Male AC and C57Bl mice were used in this study The AC strain is a hybrid line derived from the AKR/O strain and is homozygous for the adrenocortical lipid depletion gene (4) The C57Bl strain exhibits a normal pattern of adrenocortical lipids

The animals were kept under standard housing and dietary conditions (15)

Four experimental groups are included

C57Bl mice given no treatment

C57Bl mice given 0 1 mg of dexamethasone (0 025 ml of Decadron MSD") subcutaneously every second day for 3 weeks prior to death from the age of 57–59 days

C57Bl mice given 1 8 i u of ACTH (0 03 ml of Jaton prolongatum A L) subcutaneously every day for 3 weeks prior to death from the age of 57–59 days

AC mice given no treatment

The last dose of dexamethasone was given 2 hours *before death* and the last dose of ACTH 1 hour before death

All animals were killed by cervical dislocation at the age of 78–80 days

Body weight was recorded at the start of medication and immediately before killing The animals of the C57Bl groups were selected at 57–59 days in such a way that the mean body weight of the groups was as similar as possible At the time *of sacrifice the difference in body* weight of untreated AC and C57Bl mice corresponds to the findings in larger series of AC and C57Bl animals compared at the age of 78–80 days (15) Body weight and number of animals in the different experimental groups are given in Table 1

Immediately after the animals had been killed both adrenal glands were *dissected* free from periadrenal fat under the dissection microscope and fixed

The width of the cortical zones was studied in the right adrenal gland and the size of nuclei and nucleoli were studied *in the left gland.*

Measurement of zonal width Right adrenal glands were fixed for 4 days in Baker s formaldehyde solution They were embedded in gelatine and cut serially on a *freezing microtome at 10 microns The sections* were cut parallel to the longest axis of the organ floated on water stained with Mayer s haemalum for 10 minutes and mounted in Aquamount All steps were standardized as far as possible

TABLE 1

Number of Animals and Body Weight in the Various Groups

Animals for measurement of adrenal cortical width in right adrenal gland

Groups	Number of animals	Body weight at 57–59 days Mean	Range	Body weight at 78–80 days Mean	Range
Untreated C57Bl mice	19	21 8	17 5–26 0	24 9	21 5–27 5
Dexamethasone treated C57Bl mice	10	21 7	20 0–24 5	21 6	19 5–24 0
ACTH treated† C57Bl mice	10	21 6	17 0–24 0	23 2	19 0–24 5
Untreated AC mice	20	–	–	22 7	17 5–26 5

Animals for measurement of size of adrenocortical nuclei and nucleoli in left adrenal gland

Groups	Number of animals	Body weight at 57–59 days Mean	Range	Body weight at 78–80 days Mean	Range
Untreated C57Bl mice	10	21 5	18 5–25 0	24 2	20 0–27 0
Dexamethasone treated C57Bl mice	10	21 7	20 0–24 5	21 6	19 5–24 0
ACTH treated† C57Bl mice	10	21 6	17 0–24 0	23 2	19 0–24 5
Untreated AC mice	10	–	–	24 0	23 0–26 5

0 1 mg of dexamethasone s c every second day for 3 weeks

† 1 8 i u of ACTH s c every day for 3 weeks

The section having the largest medullary area the middle section was chosen for measurement of zonal width Two zones were measured the *zona glomerulosa* and the *zona fasciculata* the latter including all cortical tissue from the *zona glomerulosa* to the *medulla* The *zona reticularis* in mice is poorly defined (9) and was not measured separately

Measurement of cortical cell nuclei and nucleoli The left adrenal glands were placed in a drop of Carnoy s fluid (alcohol chloroform acetic acid 6 3 1) under the dissection microscope and cut in halves They were then fixed in freshly pre pared Carnoy s fluid for 2 hours dehydrated and embedded in histowax Sections from the central part of the glands were cut at 7 microns stained with haematoxy lin eosin and mounted in Canada Balsam All steps were standardized as far as possible When sections stained by this technique are studied with suitable filters the nucleolus shows a faintly reddish colour and can readily be distinguished from the more basophilic nucleolus associated chromatin

By changing the focus of the microscope a series of optical cross sections through the preparation are seen The area of the largest optical cross section of each cell nucleus was measured by the recording microplanimeter designed by *Caspersson Caspersson & Lomakka* (6) and taken as the measure of nuclear size An identical procedure was used for measurement of the nucleolus The standard deviation of repeated measurements was 2 0 per cent for nuclei and 6 2 per cent for nucleoli This is roughly consistent with previous investigations reported by others (8) using the same instrument

Only nuclei with one nucleolus were measured Pilot studies showed that the number of multinucleolar nuclei did not exceed 2 2 per cent in any cortical zone in any experimental group

Measurements were performed at three levels of a randomly chosen sector of the adrenal cortex The *zona glomerulosa* and the outer and the inner *zona fasciculata* The measurements in the outer *fasciculata* were done within the 5 outer layers of cells which form definite columns and this area was designated *zona fasc externa* The measurements in the inner *zona fasciculata* were done within layers of cells nearest to the medullar capsule" cells and this area was

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Numerous investigations have shown that variations in the pituitary stimulation of the adrenal cortex are reflected in the width of the different cortical zones and in the size of cell nuclei and nucleoli (11, 12, 13, 16, 22, 23). In the present work these parameters have been used to test whether the permanent adrenocortical lipid depletion is a direct result of an altered pituitary influence upon the adrenal cortex. To this end untreated AC males were compared with untreated, ACTH-treated and dexamethasone-treated C57BL males. Dexamethasone is a well known depressor of pituitary ACTH secretion.

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Male AC and C57BL mice were used in this study. The AC strain is a hybrid line derived from the AKR/O strain and is homozygous for the adrenocortical lipid depletion gene (4). The C57BL strain exhibits a normal pattern of adrenocortical lipids.

The animals were kept under standard housing and dietary conditions (1). Four experimental groups are included:

C57BL mice given no treatment.

C57BL mice given 0.1 mg of dexamethasone (0.02 ml of Decadron, MSD) subcutaneously every second day for 3 weeks prior to death from the age of 57 [illegible] days.

C57BL mice given 1.8 IU of ACTH (0.03 ml of [illegible] prolongatum AB) subcutaneously every day for 3 weeks prior to death from the age of [illegible] days.

AC males given no treatment.

The last dose of dexamethasone was given 2 hours before death and the last dose of ACTH 3 hours before death.

All animals were killed by cervical dislocation at the age of 78–80 days.

Body weight was recorded at the start of medication and immediately before killing. The animals of the C57BL groups were selected at 57 [illegible] days in such a way that the mean body weight of the groups was as similar as possible. At the time of sacrifice there was no difference in body weight of untreated AC and C57BL mice, corresponding to findings in larger series of AC and C57BL animals compared at the age of 78–80 days (1). Body weight and number of animals in the different experimental groups are given in Table 1.

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Fig 2

Nuclear size (μ^2) in the adrenal cortex Each symbol represents the mean of 20 nuclei in one animal

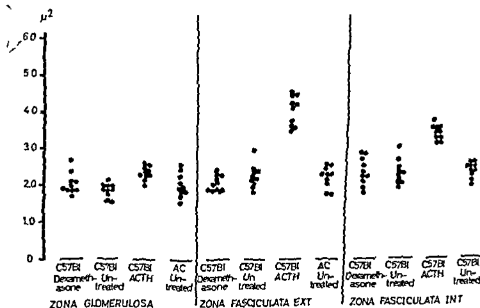


Fig 3

Nucleolar size (μ^2) in the adrenal cortex Each symbol represents the mean of 20 nucleoli in one animal

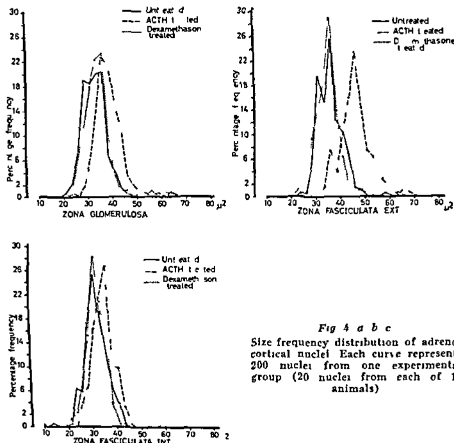


Fig 4 a b c

Size frequency distribution of adrenocortical nuclei Each curve represents 200 nuclei from one experimental group (20 nuclei from each of 10 animals)

curve for the pool of nuclear measurements showed nearly identical patterns (Fig 5)

In dexamethasone treated C57Bl animals the mean nuclear size was *not significantly different from* that *in untreated C57Bl controls in* any zone (Table 3) However as shown in the frequency distribution curve in the dexamethasone treated group a few nuclei of the *zona glomerulosa* had a size substantially greater than that observed in the control group (Fig 4)

The ACTH treated animals showed a highly significant increase in nuclear size in all zones (Table 3 and Figs 2 and 4) The increase is most marked in the outer fascicular zone (29 per cent increase) but even the *zona glomerulosa* showed considerable increase (18 per cent) The nuclei of the inner fasciculata presented an increase by 11 per cent As shown in Fig 4 the pattern of the size frequency distribution remains essentially unchanged but the whole curve is shifted to the right probably reflecting a general increase in size of all nuclei of the zones

μ²

48
44
40
36
32
28
24
20
0

C57Bl Dexamethasone | C57Bl Untreated | C57Bl ACTH | AC Untreated | C57Bl Dexamethasone | C57Bl Untreated | C57Bl ACTH | AC Untreated | C57Bl Dexamethasone | C57Bl Untreated | C57Bl ACTH | AC Untreated

ZONA GLOMERULOSA ZONA FASCICULATA EXT ZONA FASCICULATA INT

Fig 2

Nuclear size (μ^2) in the adrenal cortex Each symbol represents the mean of 20 nuclei in one animal

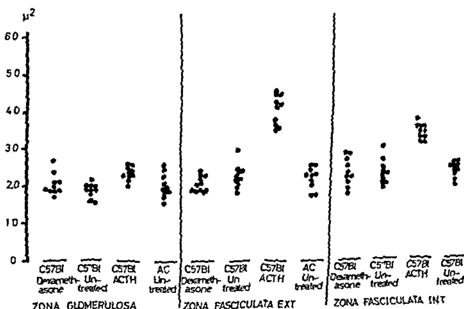


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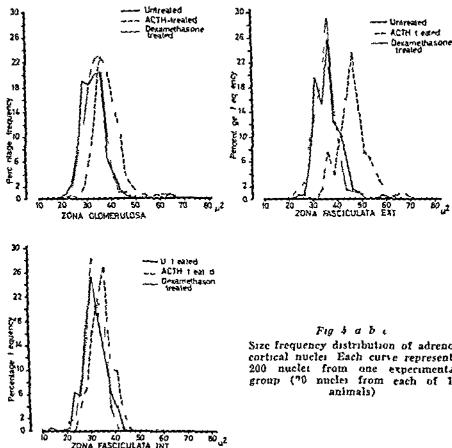
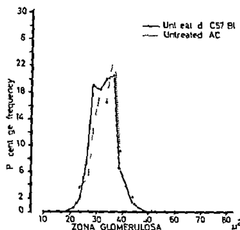


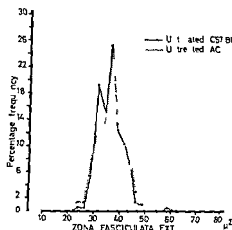
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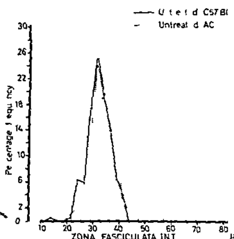


Fig a b c

Size frequency distribution of adrenocortical nuclei Each curve represents 200 nuclei from untreated AC and C 7Bl mice (20 nuclei from each of 10 animals)

Nucleolar Size

The size of cell nucleoli in the adrenocortical zones measured by maximum cross sectional areas is given in Table 4 and Figs 3 6 and 7

In untreated C57Bl animals the relative nucleolar size in the different cortical layers showed a pattern different from the relative size of nuclei The largest nucleoli were found in the inner fascicular zone and were on the average 25 per cent larger than those of the glomerular zone The nucleoli of the outer fascicular zone were 22 per cent larger than the nucleoli in the *zona glomerulosa*

Again the untreated AC mice did not differ significantly from the untreated C57Bl group in any zone (Table 4) The size distribution curves also followed the same pattern (Fig 7)

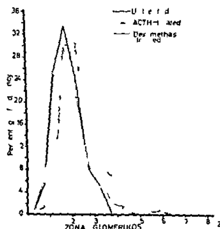
In the dexamethasone treated group the mean values did not differ significantly from those in untreated C57Bl mice Corresponding to the observations on nuclear size the size distribution curve showed that a

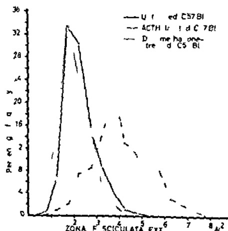
TABLE 4

The Nucleolar Size (Maximum Optical Cross Sectional Areas in μ^2) in the Adrenal Cortex of the Various Groups

Groups	Zona glomerulosa			Zona fasciculata ext			Zona fasciculata int		
	Mean	S D	P	Mean	S D	P	Mean	S D	P
Untreated C57Bl mice	1 89	0 19	–	2 30	0 31	–	2 37	0 33	–
Dexamethasone treated C57Bl mice	2 06	0 29	N S	2 0	0 21	N S	2 40	0 37	N S
ACTH treated C57Bl mice	2 36	0 18	<0 001	4 11	0 40	<0 001	3 48	0 19	<0 001
Untreated AC mice	2 07	0 31	N S	2 2	0 29	N S	2 39	0 20	N S

S D Standard deviation
P Significance of difference from untreated C57Bl group
N S Not significant





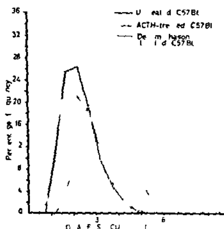


Fig 6 a b c
Size frequency distribution of adrenocortical nucleoli Each curve represents 200 nucleoli from one experimental group (20 nucleoli from each of 10 animals)

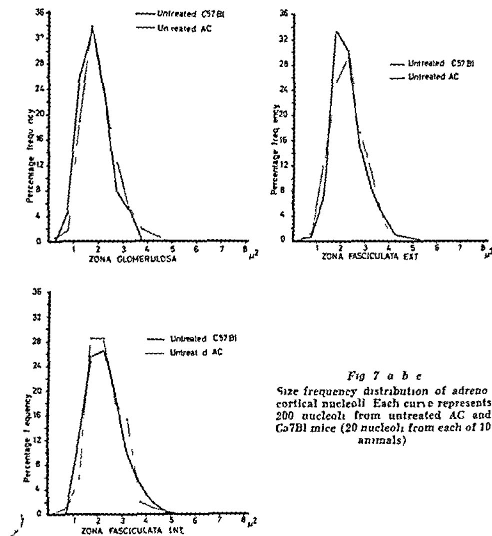


Fig 7 a b c
Size frequency distribution of adreno cortical nucleoli Each curve represents 200 nucleoli from untreated AC and C57Bl mice (20 nucleoli from each of 10 animals)

small part of the cell population in the glomerular zone had nucleoli that were substantially greater than those observed in untreated animals (Fig 6) These nucleoli belong to the enlarged nuclei described above

The ACTH treated animals showed highly significant increase in nucleolar size in all cortical layers The increase in the *zona glomerulosa* was 25 per cent in the outer fascicular zone 79 per cent and in the inner fascicular zone 47 per cent Thus the relative increase in nucleolar size was greater than that in nuclear size In contrast to the changes of nuclear size a marked increase in nucleolar size was found in the inner fascicular zone

DISCUSSION

The main purpose of the present investigation was to find evidence of the level of pituitary stimulation of the adrenal cortex in a mouse strain with spontaneous adrenocortical lipid depletion (AC)

The AC mice are strikingly similar to the untreated C57Bl mice with respect to all parameters examined except for the lipid depletion it self On the other hand both nuclear and nucleolar size and zonal width undergo marked and significant changes with experimentally induced alterations in the level of ACTH stimulation in the C57Bl strain Previous studies have shown that identical treatment with ACTH increases the adrenal weight by 68 per cent and that the dexa methasone treatment lower the adrenal weight by 23 per cent These experiments were also carried out in the AC mice and the results were almost identical in the two strains (15)

The present histometric studies give no evidence of an abnormal level of pituitary adrenocortical stimulation in AC mice It may be pointed out however that the experimentally induced alterations in the control strain as a basis for comparison are not necessarily repre sentative of mild and chronic *endogenous* changes in pituitary function As regards induction of *decrease* in pituitary function chemical depres sion with a potent gluco corticoid (dexamethasone) was considered to represent a more lenient procedure than hypophysectomy The results in the dexamethasone series may be interpreted in this way The alter ations of zonal widths after dexamethasone treatment are similar to those observed in hypophysectomy experiments on mice (10) the de crease in the size of the *zona fasciculata* may be explained as an atrophy which mainly affects the cytoplasma of the cells or as a reduction in the number of cells However contrary to the results of karyometric measurements after hypophysectomy (23) the present investigation does not point to a complete cessation of stimulation of the adreno cortical cells in so far as no alterations of nuclear and nucleolar size were found in the dexamethasone series

The study by *Tonutti et al* (23) showed that ACTH treatment com parable to the present had an effect upon adrenocortical nuclear size which was similar to although not as marked as that seen in animals subjected to severe operative stress Experimental elevation of endogen ous ACTH secretion can be effected in other ways (*e g* administration of formalin exposure to cold) However according to our experiences the toleration of mice against long term treatment of this kind is very variable In spite of important disadvantages (probably unphysiological doses and intermittent effect (19)) the experimental conditions are better controlled by administration of exogenous ACTH In the present study care was taken to sacrifice the animals when the adrenal cortex was probably under full influence of the last doses of hormones (19) In pilot studies the chosen daily doses of ACTH (and dexamethasone)

had a significant effect on adrenal weight but did not represent maximal stimuli. Therefore the present hormonal findings in the C57Bl strain should be reasonably well comparable with mild and chronic endogenous changes in pituitary stimulation.

Lipid depletion of the adrenal cortex as an expression of the *acute stress reaction* seems to be combined with an increase in the size of adrenocortical nuclei and nucleoli. During this condition there was according to *Sandritter & Hubotter* an inverse relationship between the content of lipids and the size of nuclei and nucleoli in the fascicular cells of rats (16). These results seem to be comparable with the present findings after long term treatment with exogenous ACTH and support the conclusion that the lipid depletion in AC mice is not related to an abnormal ACTH stimulation.

Thus, although the specific adrenocortical lipid pattern in AC mice is clearly dependent upon an intact pituitary function (5), neither the gravimetric (15) nor the present histometric studies give any evidence of an abnormal pituitary function in these mice. The spontaneous adrenocortical lipid depletion is best explained if it were assumed to be a primary anomaly of the adrenal cortex itself or an abnormal influence from other endocrine organs, most likely the gonads. Experiments pertaining to elucidate these possibilities will be reported separately.

In the present experiments with the *control strain* the observations agree with the findings by *Tonutti* (23) that the nuclei of the *zona glomerulosa in mice are clearly ACTH dependent. It is also remarkable* that the inner fascicular zone in untreated animals had the highest mean value for nucleolar size although it showed the smallest nuclear size. Large nucleolar size is usually an indication of high protein synthesis (7) and this finding therefore supports the theory of *Symington et al.* (21) that the inner part of the fascicular zone (the *zona reticularis*) is not a senescent part of the adrenal cortex but an active tissue.

SUMMARY

Histometric studies of the adrenal cortex in mice with spontaneous adrenocortical lipid depletion are reported and compared with similar studies in a control strain of untreated, dexamethasone *treated* and ACTH treated mice (C57Bl).

As regards the width of cortical zones, the size of nuclei and nucleoli no significant differences between mice with spontaneous adrenocortical lipid depletion and untreated control mice were found. In the control strain the hormone treatment lead to marked alterations of the *parameters examined.*

It is concluded that the spontaneous adrenocortical lipid depletion is not caused by an abnormal pituitary stimulation of the adrenal cortex.

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Acta path microbiol scandinav 75 64-70 1969

The University Department of Pathology General Hospital Malmo Sweden

METASTASES TO THE PITUITARY GLAND

By

I Hägerstrand and J Schönebeck

Received 20 x 68

Metastases to the pituitary gland have been reported mainly in patients with breast cancer (1 2 3 4 5 6) But the data given are not sufficient to allow statistical analysis of the frequency of such secondary growths The purpose of this investigation was to find out whether metastases to the pituitary gland are more common in breast cancer than in cancer of other organs

MATERIAL AND METHODS

The material consisted of subjects autopsied at the University Institution of Pathology General Hospital Malmo Sweden It consisted of two series one comprising all cases of breast cancer seen between 1st January 1958 and 30th September 1965 retrospectively studied the other was a prospective study of all subjects with malignant epithelial tumours and malignant melanomas diagnosed macroscopically between 1st October 1965 and 30th September 1967 In both series histological examination comprised not only the pituitary gland but also primary tumours macroscopically suspected metastases myocardium lungs liver spleen kidneys and lymph nodes regional to the primary tumour the supraclavicular mediastinal and retroperitoneal lymph nodes Specimens not including both the neurohypophysis and the adenohypophysis were rejected

Most of the pituitary glands were divided macroscopically in a plane perpendicular to the stalk and passing through both the adenohypophysis and the neurohypophysis Each hypophysis was cut into 1–4 pieces and one section from each piece was stained with haematoxylin erythrosin

In the statistical analysis of the data the chi square method with Yates correction was used for groups of less than 5 cases

RESULTS

The results are summarized in Table 1 and Fig 1 It is clear from Fig 1 that pituitary metastases were more common in the lower age groups and in cases with multiple metastases (metastases to 5 or more organs) Statistical analysis of the data obtained in the prospective 2 year series showed that metastases to the pituitary gland were significantly more common in subjects with mammary cancer than in all those with other types of cancer taken together (0 001 > p)

No significant difference between breast cancer and bronchial cancer was found regarding the frequency of pituitary metastases (0 1 > p > 0 05) Pituitary metastases were more common in subjects with bronchial cancer than in the rest of the material after exclusion of breast cancer (0 05 > p >0 02)

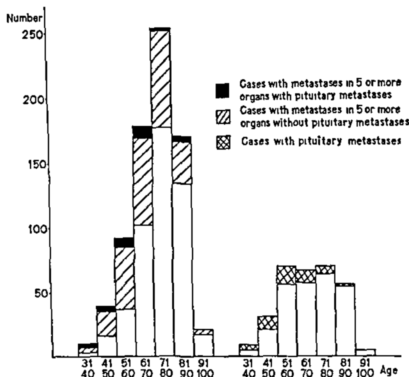


Fig 1

Age distribution of material Columns to the left represent entire tumour material on 1st October 1965 30th September 1967 Columns to the right represent age distribution of subjects with breast cancer 1958 1967

The frequency of metastases to the pituitary gland was higher among patients with carcinoma of the breast than among those with carcinoma of the colon ($p > 0\ 001$) of the stomach ($0\ 01 > p > 0\ 001$) or of the ovaries ($0\ 05 > p > 0\ 02$) while there was no significant difference in this respect between pancreatic ($0\ 1 > p > 0\ 05$) and prostatic cancer ($p > 0\ 1$)

In the subjects with metastases to 5 or more organs secondary growths in the pituitary gland were more common among those with breast cancer than in the rest of the material ($0\ 001 > p$) This was also found to be the case in subjects with skeletal metastases ($0\ 01 > p > 0\ 001$)

On careful histological analysis of the metastases in the 29 cases included in the 2 year series of malignant tumours the metastases to the neurohypophysis were found to have infiltrated the adenohypophysis or *vice versa* in 13 cases and in 7 of these the tumour grew also in the capsule In 2 of these 13 cases and only in these 2 of all 29 cases the tumour grew in the sinusoids of the adenohypophysis In 5 cases growth was seen in the capsule and in the neurohypophysis and

Acta path microbiol scandinav 75 64–70 1969

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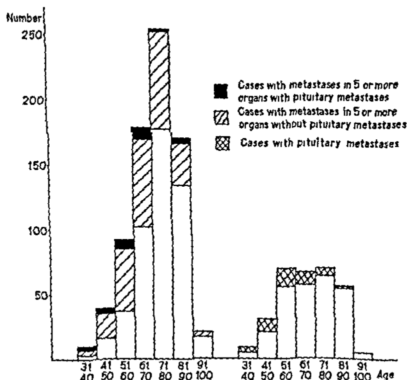


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1
in Present Material

pituitary	Number of cases with metastases to 5 or more organs		Number of cases with skeletal metastases		Number of cases with metastases to brain and/or meninges	
total (*percentage* of tumour group)	in entire tumour group	in cases with meta stases to pituitary	*in entire* tumour group	in cases with meta stases to pituitary	in entire tumour group	in cases with meta stases to pituitary
12 (12 8 %)	50	12	46	11	12	4
2 (1 3 %)	30	2	14	0	11	1
6 (5 5 %)	52	6	40	5	19	2
0	28	0	9	0	3	0
4 (8 %)	20	4	35	4	2	2
1 (2 %)	16	1	9	1	2	0
0	17	0	2	0	0	0
1 (2 5 %)	10	1	9	1	0	0
0	10	0	14	0	1	0
0	4	0	6	0	0	0
0	5	0	1	0	0	0
1	5	1	3	1	1	0
0	3	0	4	0	0	0
0	3	0	0	0	0	0
2	5	2	3	0	5	2
9 (3 8 %)	2 8	29	195	23	56	11
34 (15 8 %)						
12 (12 8 %)						
46 (14 9 %)						

breast and hypophyseal growth there were metastases to the brain and to the meninges

DISCUSSION

Since the middle of the 19th century (*Benjamin* 1857 5) many single cases of metastases to the pituitary gland have been reported (5) Publications of larger series are summarized in Table 2 (1 2 3 4 6 7)

The frequency of metastases to the pituitary gland in our cases of breast cancer was 14 9 per cent *i e* a figure roughly intermediate the highest and lowest percentages given in Table 2 In our 2 year series of malignant tumours the frequency of pituitary metastases was 3 8 per cent Statistical analysis of our findings showed that metastases to the pituitary are significantly more common in subjects with carcinoma of

TABLE 2
Survey of Published Series

Author and year	Material	Metastases to pituitary	Remarks
Wyeth 1934 (cited by Curling et al 4)	80 autopsy cases of malignant tumours	5 (6½ %)	metastases particularly to neurohypophysis
Walther 1948	3584 autopsy cases of malignant tumours	5 (1 4%) – 2 cancer of breast 1 cancer of bronchi 1 cancer of cardia 1 Ewing s sarcoma	hypophysis not routinely examined histologically
Abrams et al 1950	1000 autopsy cases of malignant epithelial tumours 16 7% cancer of breast 16 % cancer of bronchi 11 9% cancer of stomach 19 8% cancer of colon and rectum	18 (1 8%) – 15 cancer of breast (9% of cases with cancer of breast) 2 cancer of bronchi	not mentioned whether hypophysis examined histologically
Delarue et al 1964	280 malignant epithelial tumours 87 cancer of breast 8 cancer of bronchi 15 cancer of pancreas	33 (11 8%) – 23 cancer of breast (26 4% of cases with cancer of breast)	metastases particularly to neurohypophysis
Smulders & Smets 1960	71 autopsy cases of cancer of breast	20 (28 1%)	4 to adenohypophysis 7 to neurohypophysis 8 to adenohypophysis and to neurohypophysis 1 to pituitary stalk
Curling et al 1957	44 cases of cancer of breast in patients subjected to hypophysectomy	11 (25%)	metastases particularly to adenohypophysis
Duchen 1966	98 cases of cancer of breast in patients subjected to hypophysectomy	9 (9 2%)	5 to adenohypophysis and neurohypophysis 4 to neurohypophysis

the breast than among all subjects with other sorts of cancer taken together This difference had been suspected from earlier investigations (see Table 2) in which it however never had been proved statistically

The investigation showed that metastases to the pituitary were most common in subjects with widespread metastases including metastases to the vertebrae In 18 (about 60 per cent) out of the 29 cases histological analysis of pituitary metastases revealed that these metastases might have been set up by spread from secondary growths in an adjacent part of the skeleton This would help to explain why pituitary metastases were more common in subjects with carcinoma of the breast Among these subjects 52 per cent had widespread metastases (to 5 or more organs) compared with 31 per cent in the rest of the material The corresponding figures for skeletal metastases were 49 per cent and 22 per cent It is however remarkable that prostatic cancer in which skeletal metastases were more common than in breast cancer the frequency of pituitary metastases was lower in the former than in the latter group (Table 1) but $p > 0.1$ The frequency of pituitary metastases was also significantly higher among subjects with carcinoma of the breast and widespread metastases than in all other cases with widespread metastases This was also found to apply to subjects with skeletal metastases It is thus possible that additional factor are involved in the production of pituitary metastases in patients with carcinoma of the breast

SUMMARY

Routine histological examination of the pituitary at autopsy of 214 subjects with carcinoma of the breast revealed metastases in 34 cases (15 8 per cent) In order to find out whether carcinoma of the breast sets up metastases to the pituitary more often than other malignant tumours the pituitary was studied histologically in a 2 year autopsy series (763 cases) of malignant epithelial tumours and malignant melanoma Pituitary metastases were demonstrated in 29 cases (3 8 per cent) The material included 94 cases of carcinoma of the breast (not included in the first mentioned 214 cases) Twelve (12 8 per cent) of these had pituitary metastases *i e* a statistically higher frequency than in all the other cases of tumour taken together ($0.001 > p$) Pituitary metastases were most common in subjects with widespread metastases (all 29 patients had metastases to 5 or more organs) and in subjects with widespread skeletal metastases These observations could partly explain the higher frequency of metastases to the pituitary in subjects with carcinoma of the breast In the 29 cases with metastases to the pituitary gland the secondary growths in the hypophysis were confined to the neurohypophysis in 12 patients to the adenohypophysis in 4 involving both the neurohypophysis and the adenohypophysis in 13

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Acta path microbiol scandinav 75 71–84 1969

Rheumatism Research Unit of Aarhus University Kommunehospitalet Aarhus Denmark (Head H Craudal)

IMMUNOHISTOCHEMICAL DEMONSTRATION OF RHEUMATOID FACTOR (RF) IN ALCOHOL FIXED SYNOVIAL TISSUE FROM PATIENTS WITH RHEUMATOID ARTHRITIS (RA)

By

JOHANNES FRIIS

Received 23 x 68

Serological methods have for many years been used as a diagnostic aid in rheumatology Milestones are Waaler s observation of the ability of rheumatoid serum to agglutinate sensitized sheep red cells (*Waaler* 1939) and the introduction of the latex fixation test by *Singer & Plotz* (1956) Demonstration in serum of RF has weight as one criterion for the diagnosis RA (*Ropes et al* 1958) The existence of at least 3 of the following pathologicalanatomical changes shown by histological examination of the synovial membrane is also considered one criterion villous hypertrophy proliferation of superficial synovial cells infiltration of chronic inflammatory cells with tendency to form lymfoid nodules deposition of fibrin and foci of cell necrosis (Fig 1)

Mellors et al (1959) were the first to demonstrate the applicability of immunofluorescence microscopy in rheumatology claiming that the demonstration of RF in situ in the affected synovial membrane is a finding specific for RA A reliable valuation of this hypothesis would demand examination of a great biopsy material (and a clinical follow up) In this study we have tried to find a method suitable for the immunohistochemical examination The method has been used on synovial biopsies from a total of 116 patients The result will appear from the following

MATERIAL AND METHODS

Synovial tissue removed by operation partly on patients from other hospital departments partly on patients from this department, has been examined Routine histologic examination has been carried out after staining with haematoxylin and eosin Also Pappenheim staining with methyl green pyronine according to *McManus & Mowry* (1960) has been carried out An immunofluorescent analysis of the contents of RF in the synovial membrane has been made and in a few cases the contents of immunoglobulin M (IgM) and immunoglobulin G (IgG) have also been examined

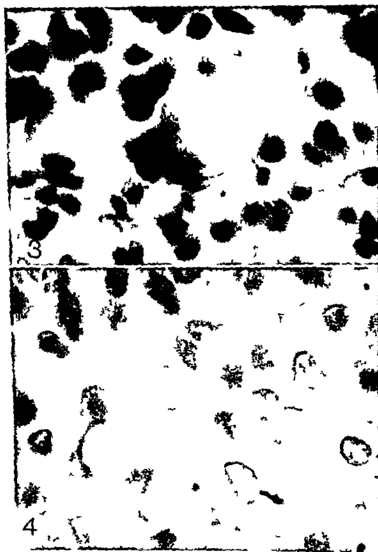
Fig 1

Nodular lymphocyte infiltrations of synovial villus exhibiting germinal centre formation Staining haematoxylin and eosin (× 100)



Fig 2

Plasmacell infiltration with many Russell bodies Staining haematoxylin and eosin (× 400)

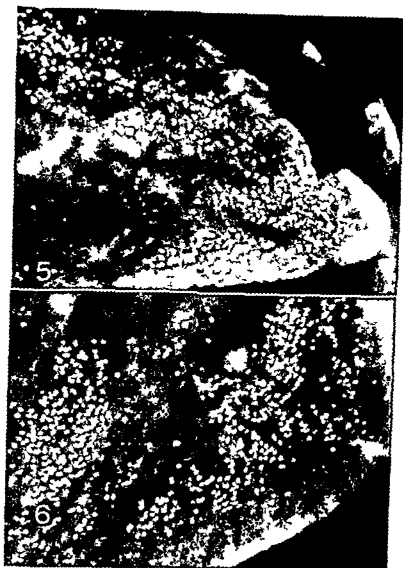


Figs 3–4

Fig 3 The most common form of Russell bodies single big globules slightly pyronin philic PAS positive and often exhibiting positive staining for RF Staining haematoxylin and eosin (× 1000)

Fig 4 Classical Russell bodies consisting of small globules in a cluster like arrangement most probably developed from a single globule as shown in Fig 3 Staining haematoxylin and eosin (× 1000)

Reagent used for the immunofluorescent demonstration of RF Aggregated human gammaglobulin conjugated with fluoresceinisothiocyanate (agg human gammaglobulin FITC) We used gammaglobulin of different origin *The results obtained* were the same whether we used 1) human gammaglobulin supplied by phil dr Albert Hansen Statens Seruminstitut Copenhagen 2) "gammaglobulin (human lyophilized) Kabi or 3) plasma fraction II (human) Hyland *Gammaglobulin*

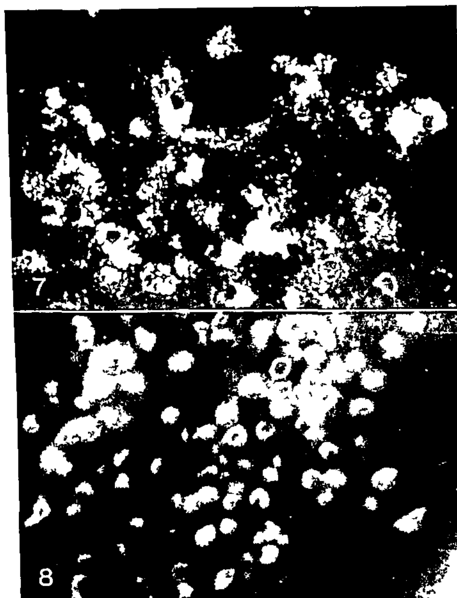


Figs 5–6

Fig 5 Numerous plasma cells just beneath the synovial cells stained for RF (from a case of active RA) Cold ethanol fixation and staining with agg human gammaglobulin FITC (× 100)

Fig 6 Plasma cells brilliant fluorescent *when stained for* IgM (case of active RA) Cold ethanol fixation and staining with goat *antihuman* IgM FITC (× 100)

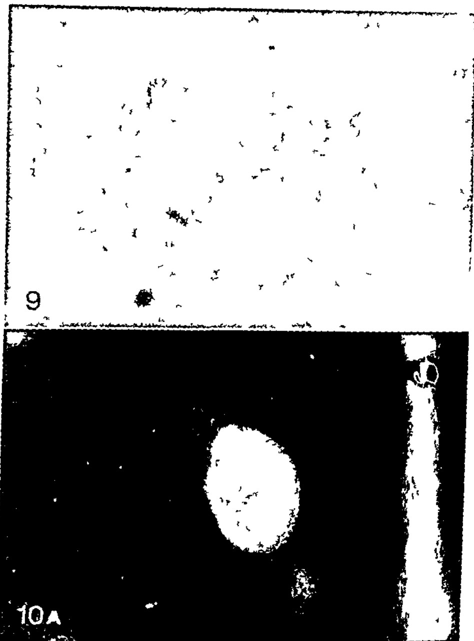
conditions In group I conventional histological examination revealed only a few anatomical pathological changes However two biopsies in the osteoarthrosis subgroup showed such changes that the ARA criterion for RA regarding histology was *fulfilled In all biopsies* rubricated in group II there was a chronic synovitis with villous hypertrophy often compact fibrin deposition and in many cases necrotic changes



Figs 7–8

Fig 7 In the cryostat technique the fluorescence is somewhat granular The sections may be cut very thin Staining agg human gammaglobulin FITC (× 400)

Fig 8 Following the Sainte Marie technique the cells are more easily recognized being sharply outlined. The fluorescence is less bright Staining agg human gammaglobulin FITC (× 400)



Figs 9–10A

Fig 9 Typical arrangement of fluorescing plasmacells in dense cell infiltration around a vessel Fixation modified Sainte Marie Staining agg human gammaglobulin FITC (× 400)

Fig 10A Single brightly fluorescent plasmacell Fixation modified Saint Marie Staining agg human gammaglobulin FITC (× 1000)



Fig 10B

Same field as Fig 10A Using a phasecontrast microsc) e ome f the morph logical details become visible f r interpretation ee Fig 10C

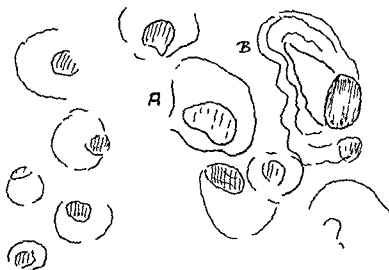


Fig 10C

Th fluore ent cell is recognized (A) and it is perhaps worth while t that it i l ing in the vicinity f a mall vessel (B)

By immunofluorescence RF was demonstrated in the plasmacells of the synovial membrane from 51 out of 64 patients with RA *i e* a positive finding in 80 per cent In 16 cases of joint diseases other than RA included in group II RF was demonstrated in 4 cases of which two clinically were rubricated as monarthritis one as dermatomyositis (sero positive with joint affection) and one as arthritis with psoriasis (sero positive) Further we have in one case of juvenile arthritis found very slight fluorescence of single plasma cells The case was classified however as negative because of the questionable fluorescence which was not reliably reproduced at a later examination RF was not demonstrated in any of the biopsies from 26 patients in the control group In all cases of positive findings RF has been localized in plasma cells or Russell bodies a fact that made the evaluation of our findings more easy

The number of fluorescent cells varied from section to section Even variation from one biopsy to another taken from the same joint from the same patient was observed

In a similar way a great variation in the number of plasma cells was found ranging from zero in some sections to an innumarable mass filling up all villies in some sections (Figs 5 9 and 10)

Among a total of 64 cases of RA (Table 1) a number of fluorescent cells was found in 10 cases varying from more than 10 up to extremely many per section In all of these 10 cases a dense infiltration with plasmacells was found in 5 of them together with more or less pronounced reaction centres In 41 cases the number of fluorescent cells varied from a single up to 10 cells per section Finally in 13 cases no fluorescent cells were found In this group (marked RF in Table 1) plasmacells were found to be almost completely absent in 4 biopsies and only two cases exhibited dense infiltration with plasmacells (one biopsy from a sero positive and one biopsy from a sero negative patient)

DISCUSSION

We found our method for conjugation of protein with FITC easy and time saving compared with the methods used elsewhere Regarding the histological technique it should be noted that previous papers concerning the immunohistochemical demonstration of RF in synovial membranes with a few exceptions have relied on observations made in synovial tissue that was deepfrozen and processed by cryostat technique *Sainte Marie* (1962) demonstrated that certain antigens resisted fixation in cold ethanol and could be demonstrated after paraffin embedding *Ball et al* (1964) called the attention to the applicability of this method for the demonstration of RF and since then Sainte Marie s technique has been used in the following two studies of the occurrence of RF in the tissues *McCormick & Hill* (1964) and *Douglas* (1965) According to *Ball et al* (1964) RF can be demonstrated even after 24

hours ethanolfixation at room temperature The modification we for practical reasons had to introduce into Sainte Marie's technique is based on this observation

To valuate the methods our results are to be compared with the results obtained by *Mellors* (1963) He and his co workers have collected the until now only greater material of synovial biopsies from patients with RA examined for the occurrence of RF The material has not been published in detail but is summed up as follows After examination of biopsies removed from at least 100 patients with different types of joint disease Mellors has in every case of active RA been able to demonstrate cells containing and presumably forming RF in lymph nodes in synovial membranes or in both Mellors found RF in biopsies removed from 5 patients with sero negative monarticular or otherwise atypical cases of arthritis All 5 patients three adults and two children eventually developed typical polyarticular RA As a control Mellors examined a biopsy material from patients with many different joint diseases other than RA in these cases he was not able to demonstrate RF in the affected synovial membrane By way of comparison in our material RF is (Table 1) demonstrated in synovial tissue from 80 per cent of a total of 64 patients with RA by examination of a single or at most two or three biopsies from every patient and from every biopsy a small number of sections This result is in reasonable accordance with Mellor's results because of the following reservations taken by Mellors Tissue in abundant quantity from every patient Mellors removed several tissue specimens and on an average cut 30–40 sections from each of the blocks prepared Secondly Mellors writes that most of the examined patients with RA had a strong positive serological reaction for RF for which reason it to some extent must be considered a selected material Personally Mellors expressed the significance of the last mentioned fact in about the following terms In cases of active classic RA in adults with a high titre of RF in serum the presence of RF will be demonstrable by examination merely of a single section from the synovial membrane whereas it may require examination of many sections to find just a few fluorescent cells when atypical and early cases are concerned

Completely necrotic biopsies were excluded from the present material (Table 1) However some biopsies consisting mostly of fibrous tissue were included and also some partly necrotic biopsies with few and pale plasmacells Such biopsies were found either to contain very few fluorescent cells or none at all Consequently biopsies containing many plasmacells are most fit for the immunohistochemical demonstration of RF containing cells

All things considered it must be reasonable to assume that we ought to have obtained a higher number of positive findings if we in every case had examined more blocks and from every block had cut more sections at suitable intervals We missed this opportunity because we

wanted to examine the applicability of the stated method for practicable screening of a greater biopsy material

In this investigation we have like Mellors been able to localize RF in plasmacells and Russell bodies in the affected synovial membranes of patients with RA Several investigators have found a similar localisation (*McCormick* 1963 *Rodman et al* 1967) *Nowoslawski & Brosko* (1967) emphasize that RF nearly always could be demonstrated in the cells of and occasionally as non cellular deposits in all reaction centres present We have observed something like this in sections from a single biopsy processed by the freezing technique but never in sections exposed to alcoholfixation As distinct from this *Fish et al* (1966) have found RF localized extracellularly partly as well defined spots (2–20 microns) partly in larger somewhat irregular areas (25–100 microns) The explanation of this discrepancy may possibly be that RF is produced in certain cells of the synovial membrane in which the concentration is highest since these cells release RF (according to Mellors by a secretion process) it may not be absurd to suggest that a high extracellular concentration might become visible if a special refined technique were used It remains to be explained why *Fish et al* failed to observe any cells containing RF

Hollander (1966) has managed to release RF from leucocytes isolated from joint effusion in cases of sero negative arthritis Morbus Still morbus Bechterew psoriatic arthritis and early stages of RA Also in these cases is RF presumably formed in the plasma cells of the synovial membrane Hollander assumes that RF reacts with IgG forming particular complexes In such cases phagocytosis by leucocytes prevents locally formed RF from reaching the circulation According to a previously advanced hypothesis (*Hollander et al* 1965) the self perpetuating joint disease is caused by release of lysosomal enzymes from the so called RA cells or ragocytes leucocytes in the joint effusion with RF containing inclusions Hollander's hypothesis might explain the improvement in the general condition of patients to be observed after synovectomy (*Laine & Vainio* 1964) immunoprocesses pernicious to the organism take place locally in or around the affected synovial membrane By removal of the immunoglobulin producing tissues the vicious circle may be broken possibly inducing a general remission

The importance which by *Hollander et al* is attributed to the local production of RF emphasizes the rationality of the examination of the synovial membrane for immunoglobulin and RF in different forms of arthritis The results of the present study will not serve to clarify the sketched problems but the technique concerned opens a possibility for further investigations which may contribute to cast light over the pathogenesis of the rheumatoid inflammation and probably conduce to a sharper distinction between different forms of rheumatism even if the finding of RF is not specific for RA

SUMMARY AND CONCLUSION

RF producing cells is an immunohistological feature claimed to be specific for the rheumatoid synovial inflammatory infiltration We have tried to develop a method feasible for immunohistochemical examination The method of choice alcoholfixation and paraffin embedding (*Sainte Marie* 1962) is a technique implying certain advantages as compared with the cryostat technique More easy dispatch of biopsies and the possibility of immunohistochemical examination and conventional staining of serial sections In this experiment we have for practical reasons ignored the temperature during fixation Even if this modification of the technique caused some decrease in fluorescence we found the method serviceable as we were able to demonstrate RF in the plasma cells and Russell bodies in the synovial membrane from 51 out of 64 patients with RA (80 per cent) and in none of the biopsies obtained from 26 patients in a control group We were able to demonstrate RF in the synovial tissue from two patients with monarthritis We find it a possibility that an examination like the one here described would be helpful in the classification of early and oligosymptomatic cases of arthritis We did not find RF in sero negative rheumatoid arthritis juvenile arthritis or psoriatic arthritis but the number of patients examined is very small For the examination of such cases the more refined cryostat technique might be preferable Further work upon this subject might contribute to a clearing up to the pathogenesis of the rheumatoid inflammation

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The importance which by *Hollander et al* is attributed to the local production of RF emphasizes the rationality of the examination of the *synovial membrane for immunoglobulin* and RF in different forms of arthritis. The results of the present study will not serve to clarify the sketched problems, but the technique concerned opens a possibility for further investigations which may contribute to cast light over the pathogenesis of the rheumatoid inflammation and probably conduce to a sharper distinction between different forms of rheumatism, even if the finding of RF is not specific for RA.

Acta path microbiol scandinav 75 85–90 1969

Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt University of Oslo
Rikshospitalet Oslo Norway

CORRODING BACTERIA FROM THE RESPIRATORY TRACT

I *Moraxella kingii*

By

S D HENRIKSEN

Received 16 x 68

An aerobic blood agar culture of pus from an abscess in an upper eyelid yielded growth of a gramnegative rod which grew as tiny shallow depressions in the agar surface together with a nonhaemolytic streptococcus Two days later a throat culture from a case of laryngitis showed an abundance of similar depressed colonies Again a couple of days later a large number of similar colonies were found in a culture of pus from an abscess in the neck This coincidence suggested that a systematic search for organisms growing with depressed colonies might be worth while

Consequently for some time all cultures from nose and throat were scrutinized very thoroughly with a magnifying glass and whenever depressed colonies were detected attempts were made to isolate them for study Three different kinds of organism were encountered which showed some form of depression of the colonies One of these which grew with hemispherical colonies which appeared to have sunk slightly down in the agar and were surrounded by shallow narrow grooves was found to be alpha streptococci and was not studied further

The other two organisms were gramnegative rods One of these which showed a distinct beta haemolysis and was identified as *Moraxella kingii* is dealt with in this paper The second organism appeared to be indistinguishable from *Bacteroides corrodens* (Eiken) and is dealt with in the following paper

A study of 437 cultures of nose and throat swabs resulted in the isolation of 18 strains of these two organisms an incidence of 4 1 per cent Among these strains 5 were *Moraxella kingii* (1 1 per cent) and 13 (nearly 3 per cent) were *Bacteroides corrodens* For several reasons it is obvious that this must be less than the true incidence 1 Some strains grow too slowly to be detected in 18–24 hour cultures and only become visible after 2 to 5 days ([illegible]) Other strains although they may be barely visible may easily be missed or may be mistaken for scratches in the agar made by the inoculating loop 3 The colonies may

be very scarce and may be hidden among other colonies in the crowded parts of the culture or may be impossible to isolate 4 Some strains may be strict anaerobes at least in the primary culture

Thus these organisms may actually be more common in the respiratory tract than suggested by these results

Although the intensive search for these organisms was discontinued other strains which were noticed while the study was in progress were isolated and subjected to a more superficial examination Altogether 6 strains of M lingu were isolated in the course of a few months

MATERIAL AND METHODS

Out of the 6 strains one (3379/67) was isolated from pus from an abscess in the upper left eyelid The other 5 were isolated from throat cultures from cases of laryngitis (3793/67) sinusitis (3974/67) chronic tonsillitis (4767/67) and rhino pharyngitis (4805/67 and 6111/67) In one of the cases (3793) very numerous colonies of the strain were found together with common throat organisms In the remaining cases only very few colonies were found together with Haemophilus influenzae in one case with pneumococci in one case and with normal throat organisms in the remaining cases

The methods were the same as those used previously in this laboratory (1 6)

RESULTS

Description of the colonies Colonies on over night blood agar cultures were very characteristic Narrow depressions of about 0 5 to 1 mm diameter with a convex smooth central papilla of a diameter corresponding to ⅓ to ¼ of the diameter of the colony (Fig 1) The papilla was surrounded by a shallow moat with finely granular bottom Apart from the haemolysis produced on bovine blood agar these colonies were like those produced by many strains of *B corrodens* The colonies were approximately circular After further incubation the colonies showed considerable peripheral spread increasing up to diameters of 4 to 5 mm In the first 2 to 3 days several concentric zones with finely granular surface were formed (Fig 2) Later after 3 to 5 days the central part of the colony gradually became filled with bacterial growth and became slightly raised with a smooth shiny (Fig 3) or an irregular surface (Fig 4) Sometimes this secondary overgrowth extended to the margin of the colony the corrosion of the agar surface only becoming visible after removal of the bacterial growth Thin granular films of growth sometimes surrounded the colonies (Fig 5) Old cul

would be very difficult to distinguish from all the small beta or alpha haemolytic organisms which are so common in throat cultures

Among 11 previously known strains as many as 8 were isolated from blood cultures or pus This may not mean that this is an organism with a high pathogenic potential The fact that it is isolated so rarely speaks against such an assumption The explanation may rather be that the organisms are very difficult to detect unless they appear in pure culture or in predominating numbers One of the 6 strains included in this study was isolated from pus from an abscess together with nonhaemolytic streptococci but there is no indication as to its aetiologic role in this condition One other strain was present in large numbers in a throat culture from a case of laryngitis but again there is little reason to think that it was the cause of disease All the other strains were very scarce in the primary cultures and probably without any pathogenic role

SUMMARY

Six strains of *Moraxella kingii* were isolated from throat cultures and from a sample of pus The strains differed from previously known strains in growing with a peculiar corroding type of colony on blood agar Some strains also occasionally produced ordinary smooth colonies The strains differed from previously known strains in a somewhat higher growth energy in being somewhat more hardy and in slightly lower levels of antibiotic sensitivity but otherwise they have the same characters as previously studied strains Available evidence suggests that this is a comparatively harmless parasite on the mucous membranes of the respiratory tract

References are given after the following paper

Acta path microbiol scandinav 75 91-96 1969

Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt University of Oslo Rikshospitalet Oslo Norway

CORRODING BACTERIA FROM THE RESPIRATORY TRACT

2 *Bacteroides corrodens*

By

S D HENRIKSEN

Received 16 x 68

As reported in the preceding paper a number of strains considered to belong to *Bacteroides corrodens* Eiken (2) were isolated from aerobic blood agar cultures of nose and throat swabs A total of 26 strains were isolated in the course of about 3 months One of these strains was isolated from an abscess in the neck together with nonhaemolytic streptococci

MATERIAL AND METHODS

Out of the 26 strains 15 were subjected to more extensive studies whereas the remaining 11 strains were examined more superficially In addition 4 strains both in the R form and in the S form were received from Dr *L Reinhold* Martin Luther Universitat Halle/Saale The 4 R strains were studied more closely whereas the S strains only were subjected to a limited number of tests Furthermore 6 of the strains studied by *Eiken* (2 3) were received from Dr *P Holm* Statens Serum institut Copenhagen Three of these strains were facultative and three strict anaerobes Only the former were studied more closely

I am grateful to Dr *Reinhold* and Dr *Holm* for the strains

The methods except where stated were the same as those used before in this institute (1 6) Three methods were used for the oxidase reaction dropping solutions of 0 5 per cent of dimethyl p phenylenediamine or of c 0 3 per cent of tetramethyl p phenylenediamine on to blood agar cultures and the method of *Kovacs* (8) in the case of the tetramethyl compound

RESULTS

Microscopy Slender mostly short gramnegative c 0 25–0 5 μ × 1–3 μ with rounded ends non encapsulated immotile without spores and without any very marked tendency to appear as diploforms or chains

Colonies As described before (2 3 5 7 10) Most often shallow depressions in the agar usually with a central papilla varying from a tiny hardly raised shiny spot in the middle of the depression to a domed papilla which fills most of the depression and is surrounded

The excellent technical assistance of Mrs *Valeria Gencay Gaeck* in this and the preceding study is gratefully acknowledged

cause of disease except possibly in one case The number of colonies varied from a single one to a large number but mostly it was small The variation in numbers did not appear to be related to the clinical diagnosis The diagnoses were mostly trivial ailments such as sinusitis pharyngitis rhinitis allergy tonsillitis etc In one case the organism was found in large numbers together with nonhaemolytic streptococci in a culture from an abscess in the neck In view of the fact that the organism has frequently been found in actinomycotic pus (3) one might suspect this to be a case of actinomycosis but no actinomyces granules were found and only one sample of pus was received so the aetiology of the abscess is unknown *Khairat* (7) *carried out animal* experiments with negative results

TABLE 1

Antibiotic Sensitivity of 23 Strains of Bacteroides corrodens *Range of Sensitivities*

Pen	Str	Chlor	Tetra	Ery
36–24 (0 03–1 0)	30–18 (0 05–8 0)	36–24 (< 0 005–0 1)	34–24 (0 02 0 4)	33–19 (0 05–8 0)

Oxytetracycline used
Results are given as zone diameters in mm and in parantheses as approximate minimal inhibitory concentrations

DISCUSSION

B corrodens has previously only been isolated from anaerobic cultures and has been considered as an anaerobe although some strains have grown aerobically in subculture This is the reason why the organism was classified as a Bacteroides species by *Eiken* (2 3) The results reported *in this paper show that this organism occurs as a facultative* anaerobe The experience of *Reinhold* (11) indicates that many perhaps all strains can be adapted to aerobic growth Some of *Eiken's* strains also grew aerobically in subculture whereas *Khairat* (7) only obtained growth under anaerobic conditions Thus it is possible that the species has both facultative and strict anaerobes *Bacteroides is* supposed to contain only strict anaerobes and it is therefore highly doubtful that this organism rightly belongs in this genus

Bacteroides appears to be a heterogeneous genus and there may be reason to doubt that the common characters of the species rodshape gramnegativity and anaerobiosis necessarily are indications of true relationship It seems probable that genera such as this one which are based upon only a few or a single criterion will come to be reclassified when more is known about their mutual relationships as shown by criteria such as DNA base composition DNA hybridization and genetic recombination In any case it would not seem to be a good idea to keep a species with many facultatively anaerobic strains in this genus The

small dimensions of the rods the fastidiousness the oxidase reaction and the antibiotic sensitivity of the strains are characters which this species shares with many members of the *Brucellaceae* and it seems that this is the right family for it It is more difficult to find a suitable genus for it It possesses many distinctive characters which distinguish it from all the genera of this family and the best solution might be to create a new genus for it However before this is done more evidence about its genetic relationships to other organisms should be gathered So far attempts to determine the DNA base composition have stranded on difficulties in gathering sufficient material for preparation of satisfactory DNA extracts But further attempts are in progress For the time being the reclassification may be postponed

The marked points resemblance with *M kingii* are worth mentioning corroding colonies oxidase reaction antibiotic sensitivity But there are also marked differences relation to oxygen morphology and haemolysis It would be premature to suggest that *B corrodens* should be placed in the genus *Moraxella* But the similarities mentioned should be kept in mind also the somewhat dubitable relationship of *M kingii* to other *Moraxella* species

Although there are some minor differences between different strains of *B corrodens* the species appears to be comparatively homogeneous and there is no reason for any subdivision of the species at the present time

The results obtained confirm that this organism is a parasite of the mucous membranes of man It is fairly common in throat cultures and occurs somewhat more rarely in the nose *Reinhold* (10) isolated strains from pathological conditions with relation to the intestine and the female genital organs which suggests that it may be an inhabitant on all mucous membranes *Khairat* (7) isolated no less than 16 strains from blood cultures taken immediately after tooth extraction in a total of 100 cases which proves that this organism must be very common in the mouth and apparently comparatively harmless

Although the organism has been isolated from pathological conditions such as actinomycosis (3) there is no evidence to show that it has any independent pathogenic faculty

It is surprising that this organism which occurs with such a high frequency in aerobic cultures apparently never before has been reported from aerobic cultures The explanation is the slow growth and extremely small size of the colonies in young cultures and the fact that the colonies which appear as tiny depressions may easily be mistaken for scratches in the agar As anaerobic cultures are usually incubated longer the colonies get time to grow to a larger size and are easier to find

It may be pointed out that none but two or three of the strains would have been noticed but for the selective attention to them

None of the previous authors has designated a type strain for [illegible]

species I propose that one of the strains on which *Eiken* based the creation of the species the strain 333/54–55, is designated as the type strain This is a typical *facultatively anaerobic strain It is being de* posited in the National Collection of Type Cultures and in The American Type Culture Collection

SUMMARY

Bacteroides corrodens is a common inhabitant of the throat and mouth and also occurs in the nose Twenty six facultatively anaerobic strains were isolated from aerobic blood agar cultures of throat and nose swabs and studied in comparison with strains studied by previous authors The strains are oxidase positive both with dimethyl and tetramethyl p phenylenediamine It is suggested that this species does not fit into the genus *Bacteroides* but should be reclassified Its taxonomy is discussed and the points of resemblance with *Moraxella kingii* are pointed out It is suggested that the reclassification should be postponed until more evidence becomes available The strain 333/54–55 studied by Eiken is designated as type strain

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Acta path microbiol scandinav 75 97–103 1969

Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt University of Oslo
(Head Professor dr med S D Henriksen) Oslo Norway

HYALURONIC ACID CAPSULE IN A *STREPTOCOCCUS FAECALIS VAR ZYMOGENES*

A Comparison with Related Mucoid Strains

By

Tom Bergan and Berit Hovig

Received 28 v 68

Hyaluronic acid production is known in streptococci belonging to *Lancefield* groups A and C As far as the authors are aware only two strains of thermoresistant streptococci with hyaluronic acid capsules have previously been reported (4 6) neither reacted with group D sera (*vide infra*) We have had occasion to examine both strains together with a mucoid laboratory strain and a recent mucoid isolate of *S faecalis var zymogenes* which produce hyaluronic acid and precipitate group D sera

MATERIALS AND METHODS

Strains Strains DH (4) and L (6) were isolated from urine and stored in freeze dried state The S strain had been maintained in the culture collection of this Institute The H strain was isolated by one of us (BH) from a case of urinary tract infection —All inoculations were done at 37 C unless differently specified

Serological identification was done with HCl extracts (5) Group A to S sera were produced by Burroughs Wellcome & Co United Kingdom An anti L strain serum was prepared according to (13) with ultrasonically disintegrated cell

RESULTS

Microscopy All strains exhibited short chains of round Gram positive cocci of ca 1 micron diameter Capsules of varying width were clearly visible by staining according to *Moller* (7)

Cultural characteristics The H DH and S strains exhibited shiny mucoid amoeboid colonies on blood agar with 1.5–2 mm diameter after 18 hours They were whitish watery semitransparent with regular borders and of soft consistency The L strain colonies were 0.5 mm in diameter but otherwise like the others (Fig 1)

From primary culture of the H strain two substrains wer[illegible]ved H[illegible] and H[illegible] At pH 7.6 and 37 C th[illegible] H[illegible] strain showed [illegible] beta haemolytic zones on bovine [illegible] human blood [illegible] lightly

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Author['s] a[dd]r[ess] Kaptein W W[illegible] og Frues B[a]kte[illegible] Institutt Rikshospitalet Oslo Norway

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TOM BERGAN and BERIT HOVIG

Received 28 v 68

Hyaluronic acid production is known in streptococci belonging to *Lancefield* groups A and C As far as the authors are aware only two strains of thermoresistant streptococci with hyaluronic acid capsules have previously been reported (4 6) neither reacted with group D sera (*vide infra*) We have had occasion to examine both strains together with a mucoid laboratory strain and a recent mucoid isolate of *S faecalis var zymogenes* which produce hyaluronic acid and precipitate group D sera

MATERIALS AND METHODS

Strains Strains DH (4) and L (6) were isolated from urine and stored in freeze dried state The S strain had been maintained in the culture collection of this Institute The H strain was isolated by one of us (BH) from a case of urinary tract infection —All incubations were done at 37 C unless differently specified

Serological identification was done with HCl extracts (5) Group A to S sera were produced by Burroughs Wellcome & Co United Kingdom An anti L strain serum was prepared according to (13) with ultrasonically disintegrated cells

RESULTS

Microscopy All strains exhibited short chains of round Gram positive cocci of ca 1 micron diameter Capsules of varying width were clearly visible by staining according to *Moller* (7)

Cultural characteristics The H DH and S strains exhibited s[illegible] mucoid amoeboid colonies on blood agar with 1 5–2 mm d[illegible] after 18 hours They were whitish watery semitransparent, w[illegible] lar borders and of soft consistency The L strain colonies were [illegible] in diameter but otherwise like the others (Fig 1)

From primary culture of the H strain two substrains were H and H At pH 7 6 and 37° C the H strain showed [illegible] haemolysis zones on bovine horse and human blood a[illegible]

Author [illegible] Kaptein W Wilhelmsen og Frues Bakt[illegible]
Blk [illegible] Oslo Norway

Fig 1
Mucoid colonies on bovine blood agar 18 hours × 10
A Strain H B Strain L



Fig 2
Haemolytic zones on human blood agar after 18 hours × 6
A Strain H+ B Strain H—

TABLE 1

Biochemical Characteristics

Reactions	H	DH	I	S
Adonitol	— §	—	—	—
Arabinose	—	—	—	—
Cellobiose	+ (1)†	+ (1)	+ (4)	+ (1)
Esculin	+	+	—	+
Fructose	+ (1)	+ (1)	+ (1)	+ (1)
Galactose	+ (1)	+ (1)	+ (1)	+ (1)
Glucose	+ (1)	+ (1)	+ (2)	+ (1)
Gas production (*Durham* tube)	—	—	—	—
Glycerol	+ (H+ 1) (H— 4)	+ (2)	+ (5)	+ (1)
Inulin	—	—	—	—
Inositol	—	+ (4)	—	—
Lactose	+ (1)	+ (4)	—	+ (1)
Maltose	+ (1)	+ (1)	+ (?)	+ (1)
Mannitol	+ (1)	+ (1)	+ (2)	+ (1)
Mannose	+ (1)	+ (1)	+ (?)	+ (1)
Melibiose	—	—	—	—
Raffinose	—	—	—	—
Rhamnose	+ (4)	+ (4)	—	+ (2)
Saccharose	+ (1)	+ (1)	—	+ (1)
Salicin	+ (1)	+ (1)	+ (4)	+ (1)
Sorbitol	+ (1)	+ (1)	+ (5)	+ (1)
Starch	+ (1)	+ (1)	+ (5)	+ (1)
Trehalose	+ (1)	+ (1)	+ (4)	+ (1)
Xylose	—	+ (5)	—	—
Arginine	+	+	—	+
Gelatin	—	—	—	—
Na hippurate	—	—	—	—
Urease (*Christensen*s agar)	—	—	—	—
Indol	—	—	—	—
Methyl red	+	+	+	+
Voges Proskauer	+	—	—	+
Citrate growth (*Koser*s medium)	—	—	—	—
KNO reduction	—	—	—	—
pH in glucose broth after 5 days	3 1	3 3	5 0	3 0
Litmus milk (Difco)				
Reduction	+ (6 hours)	—	—	+ (6 hours)
Curdle	+ (2)	—	—	+ (?)
Acid	+ (4)	—	—	+ ( )
Digestion	—	—	—	—
Triphenyltetrazoliumchloride (0 04 per cent in nutrient agar)	+	±	—	+
Catalase (3 per cent H O on slide)	—	+	—	—
Oxidase reaction (*Kovac*s method)	—	—	—	—
Soluble haemolysin ‖	—	—	—	—
Tyrosine hydrolysis (??)	+	+	+	+

In 1 per cent peptone water

§ Positive or negative reactions are indicated by + or —

† Numbers in parantheses indicate th n imber of days elapsed bef c posili e reaction

15 per cent gel t n in broth

‖ Filtrates of 24 hour *Todd Hewitt* Br th (Oxoid) growth mixed with qual parts of separate ? per cent su pensions of Fo i human and rabbit eryth ocytes

narrower on the first) whereas the H strain exhibited alpha haemolysis on bovine blood and beta haemolysis only on the horse and human blood (Fig 2) This distinction has remained after 25 subcultures

Strains DH and S behaved like H while the L strain gave alpha haemolysis on all plates The zones of clear haemolysis were wider by anaerobic than by aerobic growth

No soluble haemolysins were demonstrated against bovine human or rabbit erythrocytes

Our findings stress the paramount importance of selecting a suitable kind of erythrocytes for blood agar On bovine plates only the H strain and not the H, DH or S strains showed the beta haemolysis which is essential for a correct species diagnosis

All strains were non motile in semi solid agar and no smell was detected from blood agar growth

Biochemical reactions are recorded in Table 1 Growth was observed in all tubes with negative reactions

After 12 days growth in litmus milk potentiometric measurements showed pH = 5 9 with L pH = 5 5 with DH and pH = 5 5 with an acid producing *S bovis* from The Streptococcus Reference Laboratory London The uninoculated controls had pH = 6 2

The DH strain was much slower in forming acid from lactose than H and S but among the strains examined it was the only one to ferment inositol and xylose Strain L did not form acid from lactose rhamnose or saccharose

TABLE 2

Growth Characteristics

Tests and Media	H	DH	L	S
Heat Resistance				
Growth after 56 C for 30 min	+			+
60 min	+	+		+
Growth at 10 C	+	+		+
45 C	+	+		+
pH 9 6	+	+		+
Growth with 6 5 per cent NaCl	+	+		+
40 per cent bile §	+	+		+
0 02 per cent methyl blue in milk				
a) reduction	+	+		+
b) coagulation	+	−		+
Tellurite †	−	+		+
Growth on/in Glucose broth	+	+		+
Phosphate broth	+	+	+	+
Lactose bromothymol blue agar	+	+		+
Anaerobically ‖	+	+		+
Growth with optochin discs	+	+	+	+

Presence of or lack of growth is indicated by + or −
In Dextrose Broth (Difco)
§ In blood agar
† 0 04 per cent in McLeod's agar
Without glucose
‖ Anaerobic jar with H atmosphere

TABLE 3
Antibiotic Sensitivity

Antibiotic	H	DH	I	S
Ampicillin	15–III §	29–I	43–I	25–I
Bacitracin	17–III	17–III	39–I	22–I
Cephalosporin	8–IV	9–IV	24–I	0–IV
Chloramphenicol	23–I	30–I	40–I	30–I
Erythromycin	26–II	31–I	48–I	28–I
Lincomycin	13–III	13–III	50–I	21–II
Methicillin	0 IV	14–IV	37–I	16–IV
Neomycin	13 IV	14–III	16–II	17–II
Nitofurantoin	23–III	30 III	29–III	28 III
Novobiocin	17–II	17–II	22–I	18 II
Oxytetracycline	30–I	31–I	42–I	34–I
Penicillin	23–III	2[illegible]–III	38–I	21–III
Streptomycin	18–II	19–II	15–III	20–I

All strains were resistant to colistin kanamycin nalidixic acid polymyxin and sulphonamide
Tested with discs from Bakteriologiska Laboratoriet Karolinska Sjukhuset Stockholm
§ Inhibition zone diameter in mm is listed in Arabic numerals
Grade of sensitivity is indicated by Roman numerals
The grades used are I = highly sensitive II = moderately sensitive III = relatively resistant IV = resistant

Growth characteristics are reported in Table 2 The H DH and S strains showed the physiological properties typical of enterococci

Antibiotic sensitivity is reported in Table 3

Demonstration of the hyaluronic acid capsules The strains were non mucoid 1–1 5 cm from a streak of hyaluronidase producing *Staphylococcus aureus* (4) Similarly the hyaluronic acid producing strains had colonies of the same appearance as ordinary enterococci when grown on blood agar containing 50 mg of hyaluronidase (ex bovine testes) per liter

Serological reactions Group D sera produced immediate precipitation with both mutants of strain H *but not with any of the others All other* group sera gave no reactions

To exclude the possibility that the capsules might be responsible for false negative reactions the strains were grown on blood agar containing hyaluronidase prior to antigen preparation This caused immediate group D precipitation of the S strain extract The DH strain antigen however reacted rapidly and the I strain after 10 minutes with group C serum No change in precipitation was observed with the strains H II or a non mucoid group D strain

The Wellcome Research Laboratories Kent U K with their standard technique subsequently found that group D sera gave immediate [illegible]tions with H and S strain antigens The reaction between DH and [illegible] C serum appeared after 10 minut[illegible] whereas the I strain ga[illegible] no reaction

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b) coagulation	+	—	—	+
Tellurite †	+	+	—	+
Growth on in Glucose broth	+	+	+	+
Phosphate broth	+	+	±	+
Lactose bromothymol blue agar	+	+	—	+
Anaerobically ‖	+	+	+	+
Growth with optochin discs	+	+	+	+

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Chloramphenicol	23 I	30–I	40–I	30–I
Erythromycin	20–II	31–I	48–I	28–I
Lincomycin	13–III	13–III	50–I	21–II
Methicillin	0–IV	14–IV	37–I	16–IV
Neomycin	13–IV	14–III	16–II	17–II
Nitofurantoin	23–III	30–III	29–III	28–III
Novobiocin	17–II	17–II	22–I	18–II
Oxytetracycline	30–I	31–I	42–I	34–I
Penicillin	23–III	25–III	38–I	21–III
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Attempts to produce anti L strain antibodies did not elicit any reaction after immunization for two months and a half

Consequently the H and S strains definitely belong to *Lancefield* group D The DH strain belongs to or cross reacts with group C whereas strain L is non typable

DISCUSSION

The results indicate that the strain isolated by us H is the first hyaluronic acid producing strain readily reacting with group D sera The strain exhibited two haemolytic variants with otherwise identical properties The only difference was that the cephalosporin disc produced an 8 mm inhibition zone with H but no inhibition with H+ Metabolic properties indicate that the H strain—as well as the DH and S strains—is a *S faecalis var zymogenes*

The DH strain is not typical since the characteristic litmus milk changes did not appear and starch was hydrolyzed Its relationship to group C was rather surprising in view of the enterococcal properties The strain must be a *S faecalis var zymogenes*, however by virtue of its enterococcal attributes beta haemolysis resistance to 0 04 per cent tellurite and acid production from sorbitol

Conformity of the L strain to *S faecalis* is incomplete since only two of the hallmarks of the enterococci were present acid was not formed from lactose and it did not react with group D sera Only growth at pH 9 6 and moderate thermoresistance (surviving 56 C but not 60 C for 30 minutes) were demonstrated This strain is difficult to classify It is worth noting that this biotype was not seen by *Skadhauge* (12) There are certain similarities to *S salivarius saprofyticus* and *S salivarius septicus* of the viridans group as defined in (12) and to *S equinus* as evidenced by its lack of lactose fermentation Strain L however seems to belong to the *S sanguinus* aggregate described by *Coleman & Williams* (1) after their computerized cluster analysis on 346 alpha haemolytic streptococci

Previous reports on mucoid group D streptococci are scarce In 1938 *Dawson et al* (2) described enterococcal M forms in a thorough review on streptococci Their two M strains do not appear to be as mucoid as the H DH or S strains and no indication of hyaluronic acid production was presented *Nyman* (8) in a survey on 449 enterococci which included one of the M form *Dawson* strains was not able to identify any encapsulated strain in spite of attempts to induce capsule production by using rich media and mouse passages *Skadhauge* (11) has reported that some *S faecium* strains have small capsules of a material that give a swelling reaction with type specific sera In *S faecalis* however he could not demonstrate mucoid or encapsulated strains In a review on the taxonomy of group D streptococci *Deibel* (3) elaborated on polysaccharide and slime formation without mentioning production of hyaluronic acid production or the occurrence of mucoid strains

SUMMARY

Four hyaluronic acid producing streptococci from the urine were compared Two strains of *S faecalis var zymogenes* H and S which appear to be the first mucoid hyaluronic acid encapsulated streptococci to react with group D sera are described Another strain of this species described earlier (4) showed no group D specificity but cross reacted with group C sera A fourth strain (6) seemed more distantly related to the enterococci

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Acta path microbiol scandinav 75 104–110 1969

WHO International Escherichia Centre Statens Seruminstitut Copenhagen Denmark

ACQUISITION OF THE K 12 F MALE STATE AND THE f ANTIGEN BY *SHIGELLA FLEXNERI* STRAINS

By

I KETYI[1]

Received 6 xi 68

The study by *Luria & Burrous* (7) on the first hybridization experiments with *E coli* K–12 and different Shigella strains reported a successful transfer of the F factor However the few F Shigella cultures tested did not show an ability to chromosomal transfer Since those authors lacked a convenient simple selective method their experiments were not extended to investigate the effectiveness of F transfer

The present study provides some information concerning F transfer into strains of *Shigella flexneri*

MATERIALS AND METHODS

Strains The strains used in this study are listed in Table 1 Some polyaux trophic mutants of *Sh flexneri* were selected with the aid of an alkylating agent– Nitroso methyl carbamide (4)

Sera The standard f (W3703) and f (W1611) sera were prepared from I and F cultures of an *E coli* strain belonging to serotype O25 K H obtained from *I* and *F Orskov*

An f serum was prepared according to the method described by *Orskov* (13) using an (f) strain of *Sh flexneri* 4 b (D4101) except that formalin treated instead of live culture was used as antigen

The polyvalent and factor sera of *Sh flexneri* were prepared and provided by A Vertenyi (Institute of Microbiology University Medical School Pecs Hungary)

Antigen antibody reactions Slide agglutination tests were made with 1 10 or 1 20 serum dilutions and fresh broth agar cultures Tube agglutination was performed with twofold serial dilutions of sera and freshly prepared saline suspensions of 18 to 24 hour old broth agar cultures After 24 hours incubation at 37 C readings were made by means of a magnifying glass

The absorption of antisera was made by the method of *Orskov* (13

Phage sensitivity tests These were performed with the male specific phage f1 (12) from the stock collection of the WHO International Escherichia Centre and 18 to 24 hour old broth cultures of the strains examined Strain W1876 was used as control The phage dilutions were made in *Loeb* solution (8)

Media The minimal medium used in this study was prepared according to *Falk* et *al* (2) but with double concentration of glucose and with an agar content of

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1 Address as from 1969 Institute of Microbiology University Medical School Pecs Hungary

2 The author wishes to express his sincere thanks to Drs *I* and *F Orskov* WHO International Escherichia Centre Statens Seruminstitut Copenhagen for their interest and for advice and criticism in connection with this study

TABLE 1
Strains Used

genus	serotype	source	strain designation	markers
F che richia coli	0 ?K –H48	WIEC	WC	F Met Mal Lac
	0 ?K –H48	WIEC	W187C	F Thr-Leu Thi fl
	0 ?K –H48	WIEC	W1607	F Met
	0100 K? H2	WIEC	D512	F His Ily-Mal
	1a	Weil[1] (63–125–700)	UP511	Nia Met Mal
	1b	Weil (6–1–409)	UP1051 ?	Nia Trp Leu Ly Arg Mal
			D1101 †	(f) derivate of UP1051
	?a	Weil (63–40)	UP512	prot trophe Mal
	?b	Weil (66–1–1208)	UP5?6	prot trophe Mal
	3	r utine strain	UP3005	His Leu M l
			D3101 †	(f) derivate f UP3005
			UP304? §	His Met Thi
				Thr Trp Mal
Shigella flexneri	4aA	Carpenter[2]	UP536	Nia Mal
		103 A (Ratcliffe)	D4501 †	(f) derivate of UP 536
	4aB	Carpenter	UP537	Nia Met Mal
		103 B (H D)	D4601 †	(f) derivate of UP537
	4b	Weil (63–143 4)	UP4009	Nia Leu Mal
			D4101 †	(f) derivate of UP4009
			UP4041 §	Nia Leu His Mal
	5	Weil (63–149 119)	UP515	?3 Mal
	6	Weil (67–104–1)	UP516	? Mal
	var X	Weil (66–1–411)	UP7013	Nia Met Phe/Tyr-Mal
			D7101 †	(f) derivate of UP7013
	var Y	Weil (63–143–4)	UP 18	? Mal

WIEC = WHO International E cher chia Centre Staten Seruminstitut Copenh gen Denmarl UP = Institute of Mi robiol gy University Me lical School Pecs Hungary d rivates of train K–1? § prepared at the UP—see text † pr pared at the WIEC in the course of this study Weil[1] = type strains of A J Weil Carpenter = type trains of P Carpenter ?3 = undetermined marl ers

per cent Amino acids were supplemented in a concentrati n f 20 $\mu g/ml$ and the thiamine concentration was 4 $\mu g/ml$

Examination of fermentative characters and th election of the F d nor and recipient were carried out on EMB plates ith a sugar content of 1 per cent except in the case of malt se where the sugar content was 1 5 per cent Strain UP537 (4 B) which was inhibited on EMB was elected on brom th mol blue plates

Ox heart broth infu i n agar (1 6 per cent ag r) was u ed as c mplete soli l med um an l beef broth a flui l me lium In b th ca es 1 per cent pept ne 0 3 per nt NaCl and 0 2 per ent Na HPO 12 H O were adde l

I nversion The meth l de l d by *Orskov* (13) was used *th ugh m lified* a r gar is ino ulati n qu ntiti Th latter must be determined *individuall* f r all trains in r ler t pr ent o g wth an l t ensure an effective F tran fer rate

Usuall 24 h ur ld mix l cultur f F l n r and F recipient were seeded n t EMB sugar pl te n l t l t 50 l nie of the recipient were tran ferre l to broth agar plate The 10 t 24 h u ld m cr olonies were tested b slide agglutination f r the pres n f f ntig n Th (f) culture were tested in f erum and *Sh flexneri* f ct r e

A ri uring Curing of the f antigen was perf rmed ac rding to *Hir ta* (3) I the cult t n f (f) tr in in erie f br th cultures (pH 7 6) without and

with increasing amounts of acriflavine (10–80 $\mu g/ml$) After incubation at 37 C for 24 hours dilutions were made from acridine broth and from control medium without acridine inoculated on to broth agar plates After 18 to 24 hours growth single colonies were tested for the presence of antigen f

Mating technique 18 to 24 hour old broth cultures of both parents were centrifuged and washed in saline The saline suspension (about 10^9 bacteria) of each parent were plated separately for reversion control Similar amounts were mixed on minimal agar plates with appropriate supplementations The recombinant colonies were purified and analysed further for unselected markers of the donor and recipient (6)

Nomenclature The nomenclature suggested by *Dem rec et al* (1) was followed

RESULTS

1 *f⁺ Antigenic Conversion*

The acquisition of the f^+ antigen was used for selection of male *Shigella flexneri* strains Representative strains of *Sh flexneri* serotypes used as recipients were examined for the appearance of antigen f after mixed cultivation with the W6 F^+ strain of *E coli* K–12 Similar experiments were made with *Sh flexneri* (f) strains as donors The Mal (f) strain D 4101 (*Sh flexneri* 4b) was used as donor for Mal recipients When the recipients were Mal the Mal (f)$^+$ strain D1101 (*Sh flexneri* 1b) was employed as donor The results of these experiments are shown in Table 2

It will be seen from the table that f^+ antigen was found with a frequency of 1/10 to 1/100 in the colonies examined The donor/recipient ratio probably varying during the incubation period was not followed However on the basis of inoculation rate and the rates of colonies

TABLE 2

(f) Conversion of Shigella flexneri *Strains after 24 Hours of Mixed Cultivation Using* E coli *and* Sh flexneri *as Donors*

(f) type strains	E coli WF	donor strains D4101 (type 4b)	D1101 (type 1b)
1a	2/200	§	? 150
1b	3/50	6/50	
2a	3/50		2 50
2b	1/50	1/100	
3	10/50	6/100	
4aA	1/50	3/100	
4aB	6/50		9 50
4b	5/100	8/50†	
5	?/50		4 50
6	1/50	8/50	
var X	3/50	6/50	
var Y	1/50	1/100	
E coli 0100 D51?	69/100	1/50	

= number of (f) colonies/colonies examined § = not investigated
† = in this case the recipient was UP4041 a Mal mutant

observed after plating the importance of an F donor surplus in the mixed culture became apparent

The data presented here shows no differences between *E coli* or *Sh flexneri* donors nor was the result of f conversion influenced markedly *by using a pair of the same strain (Sh flexneri 4b) as recipient and* donor On the other hand the f conversion seems to be more effective in the case of transfer from K–12 to *E coli* 0100 (D512)

2 *Sensitivity to Male Specific Phage f1*

The sensitivity of representative f( ) derivates of *Sh flexneri* sero types were tested with the male specific phage fl (12) Strain W1876 of *E coli* K–12 served as control

Most of the strains investigated show the same sensitivity to phage fl as the tester strain of W1876 (with an efficiency of plating of about 10^{12} ml) Some (f) strains agglutinated only poorly Such strains *viz* *D3101 (Sh flexneri 3) D1501 (Sh flexneri* 1a) *D4601 (Sh flexneri* 4aB) and D7101 (*Sh flexneri* var X) gave plaques that were less clear than usual This finding may be reminiscent of the observation made by *Knolle & Ørskov* (5) who by measuring the fraction of non adsorbed phage found a correlation between phage sensitivity recombinant for mation and f antigen

It can thus be concluded that the (f) *Sh flexneri* strains show a male specific phage sensitivity analogous to the male strains of *F coli*

3 *Effect of Acriflavine Treatment*

The (f) strain D4101 of *Sh flexneri* 4b was treated with acriflavine by cultivation in broth medium (pH 7 6) containing different concen trations of acriflavine A dose of 10 µg/ml acriflavine which caused only slight growth inhibition resulted in clones that had lost their f antigen to an extent of almost 90 per cent Spontaneous loss of antigen f was not observable

4 *Serological Investigations*

Tube agglutination experiments performed with unabsorbed and absorbed sera and living strains showed identity between the f antigens in strain *E coli* 0100 (f) and *Sh flexneri* 4b (f) The experimental results are shown in Table 3

5 *Chromosome Transfer Ability*

The chromosome transfer ability of some of the (f) strains of *Sh flexneri* was examined the results are shown in Table 4 It will be seen that all the *Sh flexneri* strains were F since they could act as chromo some donors Strains D3101 (type 3) D4601 (type 4aB) and D7101 (var X) showed only a very poor chromosome donor capacity It should

TABLE 3

Serological Analysis by Tube Agglutination of Antigen f Carried by Escherichia coli *(strain D663) and* Shigella flexneri *(strain D4101)*

Sera	absorbed with	reciprocal agglutination titres with living E. coli O100 D663(f+)	D 12(f−)	Sh. flexneri 4b D4101(f+)	UP4009(f−)
W3703 f (O75)	unabsorbed	1280	0	5120	0
	D4101	0	0	0	0
W1611 f (O25)	unabsorbed	0	0	0	0
D4101 f (type 4b)	unabsorbed	1280	0	20480	2560
	D663	0	0	2560	2560
	UP4009	640	0	640	0
	UP4009+D663	0	0	0	0
Polyvalent flexneri serum f	unabsorbed	0	0	1280	1280

0 means titre lower than 1/20

TABLE 4

Chromosome Transfer Ability of Some (f+) Strains of Shigella flexneri

Shigella flexneri donor strains (serotype)	Recipient strains and markers selected for: UP3042 Thr	Phe	Met	Trp	D512 His	Ilv	W1607 Met
D1101 (1b)	0	0	0		+	+++	+++
D3101 (3)	0	0	+	0	0	0	0
D1501 (4aA)	0	0	0	0	+	+	+++
D4601 (4aB)	0	0		0	0		0
D4101 (4b)	+	+	+	++	0	+++	++
D7101 (var X)	0	0		+	0	0	0

0 = no recombinant + = 1-5 ++ = 5-10 +++ = 10-50 recombinants per plate
— not investigated

be noted that these strains are poorly agglutinable in f serum and have a weakly detectable male specific phage sensitivity. Strains D1101 (type 1b) and D4501 (type 4aA) show a marked chromosome transfer ability but only if the recipients used are *E. coli*. Strain D4101 (type 4b) was able to donate several chromosomal markers to the Shigella recipient strain UP3042.

Under the conditions of this study the maximal recombinant frequency was about 10

The same representatives of F⁻ Sh. flexneri types were examined for their recipient capacity. In such crosses with K–12 as donor no recombinants were detected. This is in contrast to the crosses between K12 F⁻ and the (f⁺) Sh. flexneri strains.

DISCUSSION

Eleven years ago it was shown by *Luria & Burrous* (7) that the male state could be carried over from the Escherichia K–12 system to Shigella strains.

In the present investigation it was demonstrated that any of the *Shigella flexneri* serotypes could be converted to the F⁺ state in the sense that they acquired the determinant of the f antigen, became sensitive to phage fl and in the cases tested also became capable of chromosomal transfer. The efficiency of the conversion was low, which is not unusual when the F factor is transferred among non homologous strains *Ørskov* (14), *Mäkelä* (9). Thus this difference in F conversion effectivity is not necessarily an expression of differences between the Escherichia and Shigella groups but is more likely based on unknown strain differences.

Serological analysis has shown that the f antigens found in different *Escherichia coli* serotypes infected by the K–12 male factor are identical. In the present investigation it was shown that such identity is also found when this factor is transferred to *Shigella flexneri* strains. In contrast to these findings antigenic relationship but not identity was found between the f antigens of Salmonella and *E. coli* K–12 (11).

Luria & Burrous (7) in their limited experiments were not able to show chromosomal transfer directed by Shigella strains even though they foresaw that such transfer might be possible. *Schneider & Falkow* (10) produced a *Shigella flexneri* Hfr strain by the terminal selection method which carried not only the F factor but in addition a piece of the *E. coli* chromosome. Investigations at present in progress will show whether it is possible to select Shigella Hfr strains from the donor strains detected in this study.

SUMMARY

In mixed cultures of the K–12 strain W 6 F⁺ and a series of representative *Shigella flexneri* strains successful f antigen conversion was demonstrated in all cases. About 1 to 10 per cent of the re-isolated recipient colonies has acquired the antigen. This frequency was not increased in experiments using (f⁺) *Shigella flexneri* strains as donors.

The strains in which the f antigen was produced were sensitive to the male specific phage fl and they lost the f antigen after treatment with acriflavine. All (f⁺) strains tested for the ability to chromosomal transfer by conjugation were positive in this respect. Thus the (f⁺)

Shigella flexneri strains seem to have acquired the distinct properties of maleness known to be endowed by the F factor

The f antigen carried by *Escherichia coli* was proved to be identical with the f+ antigen produced by the *Shigella flexneri* strains

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The National Bacteriological Laboratory Stockholm Sweden

INVESTIGATIONS ON THE ENZYMES AND TOXINS OF STAPHYLOCOCCI

Studies on Nuclease

By

M O Tirunarayanan G Lundblad and H Lundbeck

Received 7 vi 68

Cunningham, et al (9) demonstrated the occurrence of a deoxyribonuclease (DNase) in staphylococci in 1956 This enzyme was of particular interest to biochemists as it markedly differed from DNases from other sources Staphylococcal DNase was found to degrade deoxyribonucleic acid to yield on exhaustive digestion only nucleotides bearing a 3 monophosphate terminal The enzyme was thus useful to distinguish nucleic acid residues having either 5 or 3 phosphate terminals (8 15 16 18) Methods for the purification and crystallization of DNase were described (1 7 15 17 19) and studies of the amino acid composition and sequence were made (2 3 20 21) The DNase of staphylococci was found also to be active on ribonucleic acid (3 8 18 20) and thus could be termed as a nuclease without apparent specificity to the sugar moeity

In addition to the properties outlined above staphylococcal nuclease had other applications For instance it was claimed that the enzymatic nuclease test was as sensitive as the serological test for detection of enterotoxin A used for the demonstration of staphylococcal food poisoning (5) The heat stability of nuclease made it possible to reveal its presence in boiled or heat sterilized food thus permitting detection of staphylococcal contamination prior to such heat treatment On the other hand the nuclease was also found to be produced in close association with coagulase by clinical strains It was therefore suggested that the nuclease test might be applied for the detection of potentially pathogenic staphylococci (27) A similar correlation between the production of nuclease and coagulase was also demonstrated by *Burns & Holtman* (4) Experiments were therefore carried out to study the occurrence of nuclease in clinical strains and to purify the enzyme

The authors express their gratitud t D ent *Eskil Hultin* fo stimulating discussions and f r advice in the preparat n of this manuscript

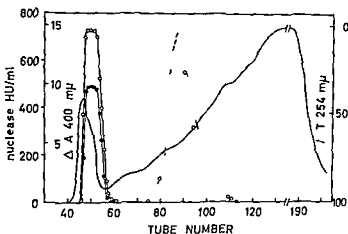


Fig 2

Separation of nuclease from phosphatase by gel filtration on Sephadex G 100 ——— per cent transmission at 254 mμ ○---○ nuclease activity (H U /ml) phosphatase (Δ A 400 mμ) at pH 5 25 ○——○ and at pH 6 90 ●——●

fer of pH 7 6 and reprecipitated with ammonium sulphate The precipitate was redissolved in 30 ml of the buffer solution

Separation of nuclease from phosphatase by gel filtration on Sephadex G 100 The solution after the second ammonium sulphate precipitation was chromatographed on a column of Sephadex G 100 with a gel bed size of 5 cm diameter and 98 cm length 0 05 M Tris HCl buffer of pH 7 6 was used The effluent was monitored through a Uvicord I absorptiometer (LKB Produkter Stockholm) measuring the absorption at 254 mμ and then collected in 10 ml lots in an automatic sample changer

The separation pattern (Fig 2) indicates the presence of a number of components of different molecular sizes Tests for phosphatase at pH 5 25 and 6 90 (Δ A 400 mμ) on the contents of the various tubes showed that the activities were contained in a single fraction eluted immediately after the first peak between tubes 46 and 59 The content of tubes 49–52 showed the highest activity No phosphatase activity was detectable in the other tubes Tests for nuclease showed that this enzyme appeared in tubes 75–111 and was thus well separated from phosphatase The contents of tubes 83–95 gave more than 50 per cent of the nuclease activity

Chromatography of nuclease on DEAE Sephadex A 50 The pooled nuclease fraction from the gel filtration on Sephadex G 100 was precipitated by full saturation with ammonium sulphate The precipitate which floated on the surface was scooped off redissolved in 0 02 M Tris HCl buffer of pH 8 0 and desalted using a 2 5 cm diameter and 10 cm long Sephadex G 25 column and the same buffer The first fraction containing the nuclease was chromatographed on DEAE Sephadex A 50 with a gel bed size of 2 5 cm diameter and 25 cm length 0 02 M Tris HCl

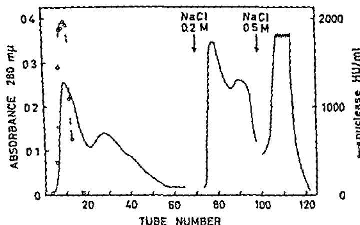


Fig 3

Chromatography of nuclease fraction on DEAE Sephadex A 50

——— absorbance at 280 mμ O——O nuclease activity (H U /ml)

buffer was used and the effluent was collected in 8 ml lots Elution was made stepwise in two stages using 0 2 M and 0 5 M NaCl in the buffer Absorption at 280 mμ on the effluent solutions gave the separation pattern shown in Fig 3 Two peaks went unadsorbed through the column two peaks were eluted with 0 2 M NaCl and the rest with 0 5 M NaCl Tests for nuclease showed that the contents of tubes 6–16 representing part of the unadsorbed first peak contained all the activity The pooled nuclease fraction was saturated with ammonium sulphate redissolved in 1 ml of buffer and desalted on a 5 cm long column of Sephadex G 25 6 ml of the nuclease containing 19 000 Hultin units per ml was obtained This preparation was used to study the different properties of nuclease

TABLE 1

Recovery of Nuclease during Purification

Step	Volume ml	Activity HU/ml	Total activity HU	Recovery per cent
Culture supernatant	2000	428	856 000	100
Sephadex G 100 [illegible] 80–100 [illegible] precipitate [illegible]	44	10910	4[illegible]0 304	56
Sephadex DEAE A 50 (tubes [illegible])	88	2600	228 800	27
Precipitation [illegible] desalting [illegible] Sephadex G 25	6	19000	114 000	13
Isoelectric [illegible] ing (0 4 [illegible] ed)	19	995	5 60	10

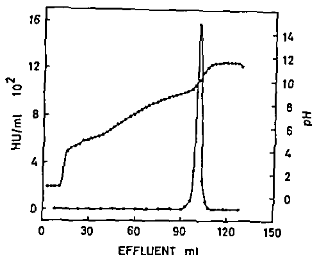


Fig 4

Isoelectric focusing of nuclease fraction from A 50 column ○—○ nuclease activity ●—● pH of effluent

Yield of nuclease during fractionation As shown in Table 1 2000 ml of the culture supernatant containing 428 HU/ml was used as the starting material The two ammonium sulphate precipitations chromatography on Sephadex G 100 gel and subsequent ammonium sulphate precipitation yielded 44 ml of the solution having 10916 HU/ml and representing 56 per cent of the starting material Desalting on Sephadex G 25 and chromatography on DEAE Sephadex A 50 gave 88 ml with 2600 HU/ml with a yield of 27 per cent A final reprecipitation with ammonium sulphate followed by desalting on G 25 gave 6 ml having 19000 HU/ml and representing 13 per cent of the original activity

Isoelectric point A method of isoelectric focusing using a mixture of ampholytes in a sucrose density gradient which has recently come into use was applied for the study of staphylococcal products (25 26) The isoelectric point of nuclease was studied using an isoelectric focusing apparatus (LKB Produkter Stockholm) and an ampholyte mixture for the pH range 3–10 0 4 ml of the desalted solution containing 19000 HU/ml was applied in the middle fractions of the gradient and a current of 300 volts–3 mA was applied The vessel was maintained at +4 C by circulating glycol water from a cryostat After 48 hours run the current was cut off and the solution was collected in 2 5 ml lots in an automatic sample changer Nuclease activity and pH of the solutions were measured and the values were plotted (Fig 4) A pH gradient of 4 0–11 5 was demonstrable Nuclease activity appeared in a single fraction between sample volumes of 90 and 110 ml with the maximal activity at 100 ml corresponding to a pH of 10 1 Thus the nuclease was found to be isoelectric at pH 10 1 75 per cent of the applied nuclease was recovered Similar studies carried out with the unfractionated cul

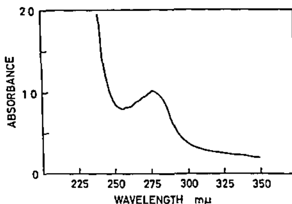


Fig 5
Ultraviolet absorption spectrum of purified nuclease

ture supernatant gave a single nuclease fraction with peak activity at pH 10 0

Absorption spectrum The ultraviolet absorption spectrum of a dilution of the nuclease in 0 2 M Tris HCl buffer of pH 8 0 was recorded using a Unicam spectrophotometer model SP 800 Maximal absorption was noted at 276–277 mμ and a minimum at 255 mμ (Fig 5) A 280/260 ratio of 1 2 was obtained

Effect of calcium and magnesium on nuclease activity Using DNA type V the effect of calcium and magnesium on the activity of nuclease was studied Different preparations of DNA varied in their content of calcium or magnesium 63 per cent of the activity was demonstrable even when no calcium was added Corresponding figures for type I was 7 per cent type III 21 per cent and type VII 68 per cent Thus EDTA had to be used to remove the contaminating anions As the concentration of EDTA was increased from 10^{-6} M to 10^{-5} M an increase in the activity above that obtained without EDTA was frequently noticed Such activation was not always demonstrable in the different batches of the substrate 5×10^{-4} M EDTA inhibited the reaction completely Using this concentration of EDTA (the effect of calcium chloride was studied Calcium concentrations above 10^{-4} M showed stimulation of activity reaching a maximum between 5×10^{-3} M and 1×10 M (Fig 6) Higher amounts of calcium inhibited the reaction

The experiments with RNA as the substrate showed that little activity occurred without addition of calcium ions The effect of calcium was therefore studied without pre incubation of RNA with EDTA 1×10^{-2} M of calcium gave maximal activity similar to that noticed with DNA as the substrate (Fig 6) Higher amounts of calcium also showed an inhibition

Experiments performed with magnesium chloride showed that magnesium was not an activator of the enzyme Magnesium h ed instead

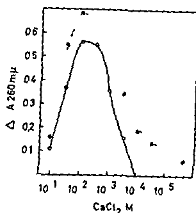


Fig 6

Calcium activation of nuclease ○—○ DNA type V ●---● RNA

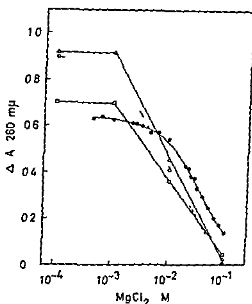


Fig 7

Inhibition of nuclease activity by magnesium

△—△ DNA type V ○---○ DNA type I □—□ DNA type III ● RNA

an inhibition of the activity when calcium was present. Up to 10^{-3} M *magnesium did not affect the activity (Fig. 7). Increase in the* concentration of magnesium above this level inhibited nuclease activity proportionally giving complete inhibition at 1×10^{-1} M. Similar results were obtained with the three DNA preparations types I, III and V studied. On the other hand RNase activity appeared to be somewhat resistant to the inhibitory effect of magnesium and complete inhibition was not attained.

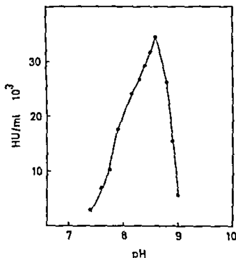


Fig 8
Nuclease pH activity relationship

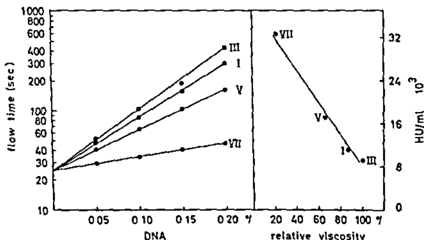


Fig 9
Viscosity of DNA and nuclease activity

pH activity relationship The effect of pH on the nuclease activity was studied using 0.1 M Tris HCl buffer for the pH range 7.0–9.2 0.1 M $NaHCO_3$–Na_2CO_3 buffer was used for the pH range 9.0–10.0 whereas 0.1 M Na acetate acetic acid buffer for the pH range 4.5–8.0 No activity was obtained below pH 7.0 and above pH 9.0 At pH values above 7.0 activity increased [illegible] with increase of pH giving a maximal value at pH 8.6 (Fig 8) Thus [illegible] pH of 8.6 was found to be optimal for the nuclease

Relation [illegible] between viscosity of substrate and nuclease activity In the study [illegible] nuclease activity by the viscosimetric method it was of

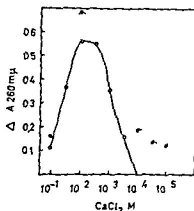


Fig 6

Calcium activation of nuclease ○——○ DNA type V ●- - -● RNA

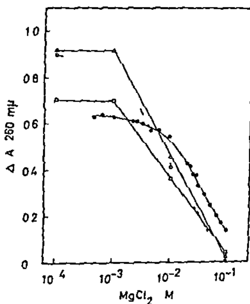


Fig 7

Inhibition of nuclease activity by magnesium

Δ——Δ DNA type V ○- - -○ DNA type I □——□ DNA type III ●——● RNA

an inhibition of the activity when calcium was present Up to 10^{-3} M magnesium did not affect the activity (Fig 7) Increase in the concentration of magnesium above this level inhibited nuclease activity proportionally giving complete inhibition at 1×10^{-1} M Similar results were obtained with the three DNA preparations types I III and V studied On the other hand RNase activity appeared to be somewhat resistant to the inhibitory effect of magnesium and complete inhibition was not attained

earlier about the occurrence of a DNase separate from RNase but it is now generally agreed that the two activities are due to the same enzyme (13)

Results reported in the present study on the separation of nuclease from the phosphatases by gel filtration on Sephadex G 100 agreed closely with similar experiments with Bio Gel P 100 described by *Sulkowski & Laskowski* (19) but not with those of *Chesbro Stuart & Burke* (6) who demonstrated the existence of multiple molecular forms of nuclease by gel filtration Isoelectric focusing also gave a single enzyme peak with maximal activity at pH 10 1 whereas *Wadstrom* (26) and *Vesterberg et al* (25) demonstrated activity in not less than five different peaks Explanations of these discrepancies probably lie in the type of strain used the method of concentration as well as the state of purity of the enzyme The nuclease is a small molecule and basic in reaction It can therefore readily complex with acidic peptides or proteins and the activity could thus be carried to different fractions during purification Thus careful consideration must be given before drawing conclusions on the significance of occurrence of either multiple molecular forms or molecules with different ionic charge

Certain interesting observations on the activity of nuclease in relation to the type of substrate used were made in the present study The occurrence of two rates of reaction as revealed by the viscosimetric assay procedure was noted with the unfractionated culture supernatants The two reactions were still demonstrable even with the purified enzyme A number of different explanations can be offered or speculated upon by which to explain the occurrence of the two rates of reactions apart from considerations of the existence of an inhibitor like magnesium in the substrate Magnesium was found to be inhibitory and occurred frequently as a contaminant together with calcium in DNA preparations Further different rates of reactions were obtained with the different DNA substrates probably due to their varying degrees of polymerization Thus these results emphasize the importance of using a well defined and purified substrate to measure nuclease activity in addition to the requirements of optimal pH and concentration of calcium ions for maximal activity

SUMMARY

The production of nuclease by 394 clinical strains of *Staphylococcus aureus* was studied Strain no 210 which produced about 400 Hultin Units per ml was used to prepare the enzyme and study its properties The nuclease attacked both DNA and RNA optimally at pH 8 6 and at a calcium concentration of 1×10^{-2} M Magnesium was found to be inhibitory giving almost complete inhibition at 1×10^{-3} M Magnesium as well as calcium frequently contaminated the DNA preparations Specific activities of the enzyme with different DNA substrates were

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The National Bacteriological Laboratory Stockholm Sweden

INVESTIGATIONS ON THE ENZYMES AND TOXINS OF STAPHYLOCOCCI

Activity of Phosphatase on Sugar Phosphates Nucleotides and Other Esters[1]

By

M O TIRUNARAYANAN

Received 20 vi 68

Results of experiments on the phosphatase activity of staphylococci using p nitrophenyl phosphate (p NPh P) as the substrate were reported elsewhere (14) Two different activities based on pH optima and metal ion activators could be demonstrated Yet the evidence of the existence of two phosphatases could not be taken as conclusive because different metal ions may activate one enzyme giving different pH optima Further these studies were carried out using p NPh P which can function as a substrate for enzymes other than phosphatase For example arylesterases are known to be active on this substrate (1) Thus a number of other phosphate esters occurring as constituents of cells had to be tried as substrates before the enzyme itself might be characterized as a true phosphatase In the present study phosphatase activity of staphylococci was examined in order to find its mechanism of action particularly as the occurrence of this enzyme has been used as a criterion by which to demonstrate the pathogenicity of the bacteria Although staphylococci have been shown to hydrolyse a number of different phosphate esters (2 3 4 6 10 11 13) a systematic study using various esters similar to previously described studies on enzymes from *Escherichia coli* (5 8) or phosphatases from other sources (12) has not been made before In addition the use of such specific substrates may aid in the separation of the two phosphatase activities noted elsewhere (14)

MATERIAL AND METHODS

Strains Three str in f *Staphyl coccus aureus* were used Two of these were linical isolates with the l b t y numbers 7 (phage pattern 75/77) and 44 (phage pattern 6/47/53) whe the th rd was a standard laboratory strain Wood 46 va iant 10344 obtain d from the National Collection of Type Cultures England These strains were previ u l found (14) to p oduce large amounts of phosphatase Th bacteria

[1] Abbre tions u ed for the esters are as outlined by the *Journal of Biolog cal Chemistry* (19(8)

were stored in the lyophilized state and taken up on nutrient agar plates before being subcultured into the medium for phosphatase production

Determination of Enzyme Activities

Phosphatase Phosphatase activity was determined by the method described by *Harper* (7) which involved the estimation of the liberated inorganic phosphate The reaction mixture consisted of

0 1 ml of enzyme preparation in buffer
0 2 ml of 0 01 M substrate solution in buffer
0 1 ml of 0 05 M $MgCl_2$ in buffer

Activity was determined in general at two pH values and the solutions were checked to have the right pH value before being added to the reaction mixture As buffers 0 2 M Na acetate – acetic acid of pH 5 75 and 0 2 *I* Tris HCl of pH 7 00 (at 25 C) were used In addition to the reagent blank two controls one without enzyme and another without substrate were included in each run Incubation was carried out at 37 C for 30 minutes after which the reaction was stopped by the addition of 0 6 ml of 10 per cent trichloracetic acid The precipitate was centrifuged off at 3000 g for 20 minutes at room temperature To 0 5 ml of the supernatant was added 2 5 ml of ammonium molybdate (0 002 M in 0 6 N H_2SO_4) and 1 0 ml of the reduction solution containing 1 amino 2 naphthol 4 sulphonic acid (0 042 M in a mixture of $NaHSO_3$ and Na_2SO_3 representing 0 56 M SO_3^{2-}) These reagents were prepared as described by *Harper* After standing in the dark for 30 minutes the absorption of the solutions at 650 mμ was measured using a Bausch & Lomb Spectronic 20 instrument with the reagent blank set at zero From a standard phosphate solution run at the same time the amount of inorganic phosphate liberated by the enzyme was calculated The values were corrected for any phosphate contained in the control solutions of the enzyme and the substrate

With a view to studying the effect of different pH on phosphatase activity the buffer solutions were replaced by physiological saline and the pH adjustment of the ice cold reaction mixture was carried out as described elsewhere (14) taking aliquots of the reaction mixture of different pH values for incubation in a water bath

A number of different phosphate esters were used as substrates The substances listed in Table 1 had a purity which according to the manufacturers varied between 94 and 99 5 per cent Reference is also made in the Table to the manufacturer by placing an alphabet after each of these substrates which is as follows (a) Fluka A G Buchs St Gallen Switzerland (b) P L Biochemicals Inc Milwaukee Wisconsin U S A (c) Hopkin and Williams Ltd Chadwell Heath Essex England (d) Mann Research Laboratories New York N Y U S A (e) Nutritional Biochemicals Corporation Cleveland Ohio U S A (f) Merck A G Darmstadt West Germany (g) Sigma Chemical Co St Louis Miss uri U S A

Coagulase Coagulase activity was determined by the method described previously (15) 0 4 ml of rabbit plasma was mixed with 0 1 ml of the test solution diluted in 0 05 *I* Tris HCl buffer of pH 7 60 (25 C) and then incubated at 37 C The highest dilution which clotted plasma after 24 hours incubation was assigned to contain one unit of coagulase activity

Protein Determination

The content of protein in the enzyme preparations was determined by the method described by *Lowry et al* (9) The proteins were first precipitated with 10 per cent trichloracetic acid washed several times by resuspending in the acid and centrifuging in order to remove traces of Tris buffer which interfered with the test

Isoelectric Focusing of Phosphatase Preparations

Enzyme preparations were fractionated by the isoelectric focusing method described by *Vesterberg et al* (16) using an LKB apparatus An ampholyte mixture for the pH range 3 00–10 00 was used 1 0 ml of the enzyme preparation was placed in the middle of the gradient and separated using a current of 300 V 4 mA for 4 hours at 4 C 2.5 ml lots of the fractions were collected in an automatic fraction collector with the aid of a tube pump pH at 25 C and enzyme activities at 37 C

were measured using these fractions Because of the low yield of phosphatase enzyme tests done with p nitrophenyl phosphate as substrate (14) are shown in Fig 10

TABLE 1

Activity of Phosphatase from Wood Strain on Various Esters

Substrate 0 005 M	Relative activity	
	pH 5 25	pH 7 00
p nitrophenyl phosphate (*c*)	100	100
phenyl phosphate (*f*)	83	100
α glycerolphosphate (*d*)	49	11
β glycerophosphate (*c*)	17	4
glucose 1 phosphate (*c*)	0	0
glucose 6 phosphate (*c*)	7	0
fructose 1 6 diphosphate (*c*)	16	2
fructose 6 phosphate (*c*)	13	1
ribose 5 phosphate (*a*)	34	9
riboflavine 5 phosphate (*d*)	40	10
flavine adenine dinucleotide (*e*)	0	0
nicotinamide adenine dinucleotide (*g*)	0	0
nicotinamide adenine dinucleotide phosphate (*g*)	0	0
adenosine 2 monophosphate (*a*)	0	0
adenosine 3 monophosphate (*a*)	12	0
adenosine 2 (+3) monophosphate (*a b*)	12	0
adenosine 5 monophosphate (*a b*)	1	0
adenosine 5 diphosphate (*a*)	7	0
adenosine 5 triphosphate (*a b*)	4	0
guanosine 5 monophosphate (a b)	*19*	*17*
uridine 2 (+3) monophosphate (*a*)	0	0
uridine 5 monophosphate (*a b*)	60	79
uridine 5 diphosphate (*a*)	65	52
uridine 5 triphosphate (*a b*)	36	[illegible]
cytidine 5 monophosphate (*a b*)	69	86
Na pyrophosphate (*c*)	14	5

p nitrophenyl phosphate being equal to 100 per cent
(a) (b) See text for name of manufacturer

EXPERIMENTAL

For the study of the phosphatase activity on different ester substrates enzyme preparations free of inorganic phosphate were needed The optimal pH values for the hydrolysis of the various esters were first determined before selection of the pH value at which to carry out kinetic studies or comparison of the activity of the enzyme on the substrates

Preparation of Phosphatase

The bacteria were grown ... feo brain heart infusion broth 500 ml of the medium ... nmeyer flasks were inoculated with 1 0 ml of a 24 hour ... strain in the same medium After 6 days growth the ... centrifuged at 3000 g for 60 minutes ... as added sodium merthiolate to a concentration of 0 02 per ... supernatant was then adjusted

to pH 3 85 using 0 1 N HCl and left to stand overnight at 4° C The precipitate that was formed was centrifuged at 3000 g and dissolved in a minimum amount of 0 05 *I* Tris HCl buffer of pH 8 00 with the aid of a few drops of 0 1 N NaOH After repeated precipitations at pH 3 85 and redissolution of the centrifuged precipitate, an enzyme *preparation free* of measurable inorganic phosphate was obtained The activity corresponded 50 60 times that of the unfractionated culture supernatant Similar preparations were made with the three strains The final solutions of the enzymes were centrifuged at *30 000 g* to remove any remaining cells n butanol was added as a preservative to a concentration of 2 per cent

Relationship of pH to the Hydrolysis of Different Esters by Phosphatase

The pH activity patterns of the hydrolysis of a number of phosphate esters by the phosphatase preparation from the Wood strain was studied using 0 005 M of the substrates and 30 *μg of* enzyme protein

Phenyl Phosphate (Ph P) and p Nitrophenyl Phosphate (p NPh P) The pH activity curves for the hydrolysis of these two substrates were essentially similar to those earlier described in the case of p NPh P (14) where p nitrophenol instead of inorganic phosphate was measured Two pH optima one between pH values of 5 00 and 5 50 and another between pH 6 50 and 7 00 were demonstrable (Fig 1)

Sugar Phosphates (Hexose and Glycerophosphate) The hydrolysis of these substances as shown in Fig 2 proceeded optimally at pH values between 5 00 and 6 00 The other optimum noticed with the

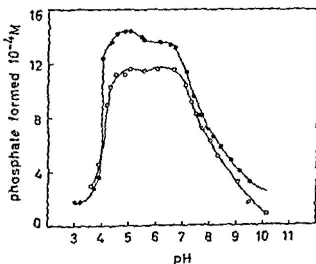


Fig 1

Effect of pH on the hydrolysis of p nitrophenyl (●——●) and phenyl (○—○) phosphates by staphylococcal phosphatase

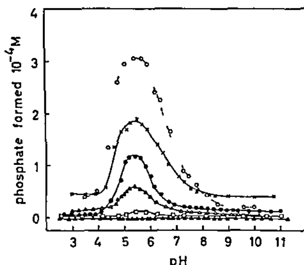


Fig 2

Activity of staphylococcal phosphatase on sugar phosphates at various pH ○---○ α glycerophosphate ×——× β glycerophosphate ●——● fructose 1 6 diphosphate △——△ fructose 6 phosphate □——□ glucose 6 phosphate ▲——▲ glucose 1 phosphate

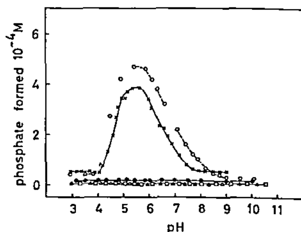


Fig 3

Hydrolysis of ribose 5 phosphate and nucleotides having coenzyme function at various pH by staphylococcal phosphatase ○---○ FMN ×——× ribose 5 phosphate ●——● NAD □——□ NADP △——△ FAD

phenyl phosphates was not demonstrable Among the different esters studied α glycerol P gave the maximal activity followed in decreasing order by β glycerol P fructose 1 6 diP fructose 6 P and glucose 6 P No activity was demonstrable with glucose 1 P

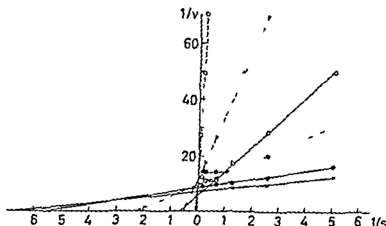


Fig 6

Lineweaver Burk plot for the evaluation of Michaelis constant for uridine phosphates O——O uridine 5 monophosphate X——X uridine 5 diphosphate ●——● uridine 5 triphosphate
Solid and dotted lines relate respectively to values at pH 5 25 and pH 7 00

These results pointed out that the diphosphate of uridine was more reactive at pH 5 25 than the monophosphate Similar experiments done with α glycerol P as substrate (not presented in figure) gave a Km value of 1 667 mM at pH 5 25

Relationship between Enzyme Concentration and Rate of Hydrolysis of Phosphate Esters

Preliminary experiments were carried out to determine the optimal concentration of the substrates to be used for a comparative analysis of the activity of the enzyme against various substrates Substrate concentrations between 0 001 M and 0 02 M were tried With many substrates 0 005 M gave the highest rates of liberation of inorganic phosphate whereas 0 001 M was insufficient and 0 02 M often inhibitory The experiments were therefore performed using 0 005 M of the substrates and the effect of varying concentrations of the enzyme was studied at pH 5 25 and at pH 7 00 The results are shown in Figs 7 and 8 The phenyl phosphates gave the highest activities at both pH values At pH 5 25 (Fig 7) the activity with phenyl phosphates was proportional to the concentration of the enzyme With other substrates the reactions were linear at low enzyme concentrations but bended off probably due to limiting substrate amount with the high amounts of enzyme Reactions with glucose 6 P showed a linear relation with enzyme concentration Similar results with the nucleotides (CMP UMP and GMP) were obtained at pH 7 00 (Fig 8)

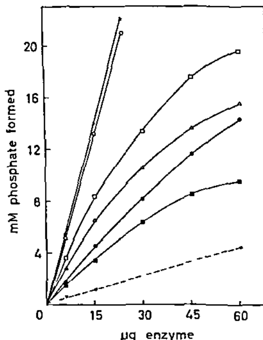


Fig 7

Rates of hydrolysis of some phosphate esters at pH 5 25 by staphylococcal phosphatase ×——× p nitrophenyl phosphate ○——○ phenyl phosphate □——□ cytidine 5 monophosphate △——△ α glycerophosphate ●——● riboflavine 5 phosphate ■——■ β glycerophosphate ×---× glucose 6 phosphate

Activity of Phosphatase on Various Phosphate Esters

Using 6 μ_g of the enzyme preparation and an incubation period of 60 minutes the activity of phosphatase on various esters was studied at both pH values as shown in Table 1 A larger number of esters were hydrolysed by the enzyme at pH 5 25 than at pH 7 00 The relative activity of various substrates are also indicated p NPh P being equal to 100 per cent Values given for the sugar phosphates at pH 7 00 are due to the high activity in the acid pH region which gives a tailing effect at this pH Thus it is not to be construed that these substances show an optimum at pH 7 00 The activity at pH 7 00 was directed predominantly on the pyrimidine nucleotides

Comparison of the Phosphatase Activity of Different Strains

The activity of the phosphatases produced by three different strains using a number of different esters are shown in Fig 9 The patterns of activity of the enzymes formed by strains nos 7 44 and Wood seemed to indicate that activity on the different substrates was similar although slight differences due perhaps to the quantity of enzyme used as well as

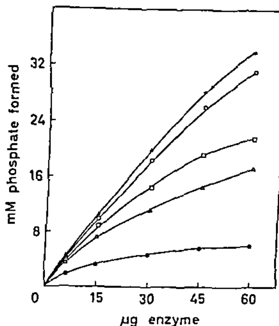


Fig 8

Rates of hydrolysis of some phosphate esters at pH 7 00 by staphylococcal phos phatase X——X p nitrophenyl phosphate ○——○ phenyl phosphate □——□ cytidine 5 monophosphate △——△ uridine 5 monophosphate ●——● guanosine 5 monophosphate

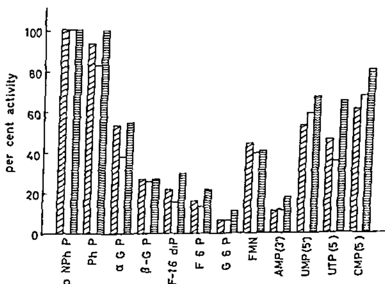


Fig 9

Comparison of the activity at pH 5 °5 against various substrates by phosphatase produced by different strains of *Staphylococcus aureus*

strain no 7 · strain Wood · strain no 44

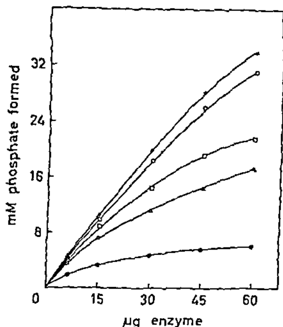


Fig 8

Rates of hydrolysis of some phosphate esters at pH 7 00 by staphylococcal phosphatase ×——× p nitrophenyl phosphate ○——○ phenyl phosphate □——□ cytidine 5 monophosphate △——△ uridine 5 monophosphate ●——● guanosine 5 monophosphate

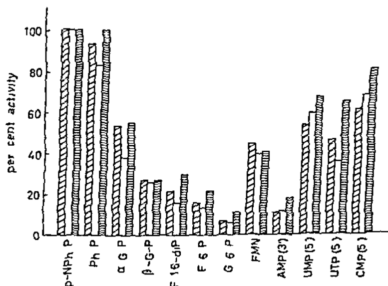


Fig 9

Comparison of the activity at pH 5 25 against various substrates by phosphatase produced by different strains of *Staphylococcus aureus*

strain no 7 strain Wood strain no 44

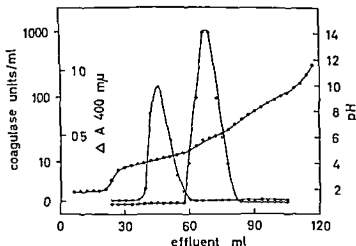


Fig 10

Separation of phosphatase from coagulase by isoelectric focusing
●——● pH of effluent ×——× phosphatase Δ A400 mμ p nitrophenol formed 30 min ○——○ coagulase units per ml

to the content of any bound magnesium could be demonstrated Similar results with the pyrimidine nucleotides were obtained when the reactions were studied at pH 7 00

Separation of Phosphatase from Coagulase by Isoelectric Focusing

Experiments were done to separate the phosphatase activities on the different substrates by isoelectric focusing Activity was determined on the fractions using p NPh P UMP α glycerol P fructose 1 6 diP and FMN During isoelectric focusing the occurrence of a broad band of precipitate was noticed which lagged behind during the collection of the samples It was found that this precipitate contained *all* the phosphatase activity With smaller amounts of the test sample the precipitate could be collected without any apparent lagging behind and thus represented the region close to the isoelectric point The phosphatase activity was found to occur between fractions having pH 3 80 and 4 20 (Fig 10) whereas coagulase appeared in the fractions with higher pH values (pH 5 90–6 10) 80 per cent of the applied coagulase was recovered but 90 per cent of the phosphatase activity was lost during the fractionation Frequently the splitting of the phosphatase precipitate into two fractions could be visualized but they could not be collected separately Other procedures are needed to find whether the phosphatase activity on the different substrates may be separated from one another

DISCUSSION

According to the results obtained in the present study, a characteristic pattern of activity of staphylococcal phosphatase on various esters can be observed. Apart from the high activity on phenyl phosphates, the enzyme is more active on pyrimidine than on purine nucleotides or sugar phosphates and thus differed from the enzymes of *Escherichia coli* (5, 8) which were found to be more active on purine than on pyrimidine nucleotides. Unlike the preparations from coli bacteria, inorganic pyrophosphate was hydrolysed by the staphylococcal enzyme.

The results obtained when the glycerophosphates were used as substrates are in close agreement with similar results obtained by *Paget & Vittu* (11). These workers also obtained a similar pH optimum (pH 5.60–7.00) and a reactivity with α-glycerol P that was greater than that given by β-glycerol P. *Ohsaka et al* (10) found similarly activity on thymidine phosphates to be greater than that on adenosine di- or triphosphates. These authors also demonstrated that staphylococcal phosphatase hydrolysed with equal efficiency both ribo- and deoxyribonucleotides. The results obtained in the present study also point to the ability of the enzyme to hydrolyse many sugar phosphates. However, from the quantitative data a certain specific relation between structure of the sugar phosphate and enzyme activity can be seen. Activity towards sugar phosphates with smaller number of carbon atoms is more marked than activity towards those with larger numbers of carbon atoms. Further, the phosphate group located on the least substituted carbon atom seems to be preferentially attacked. Thus α-glycerol P is more active than the pentose or hexose phosphates and also β-glycerol P. Substitution on the phosphate group (NAD, NADP or FAD) renders the substrate inactive but a free phosphate (FMN) gives as good an activity as the pentose P (ribose-5-P). Further work with several derivatives are needed before any specific relationship between structure of substrate and enzyme activity can be established.

The spectrum of activity against different phosphate esters was quite similar when the three enzyme preparations were used. These results indicated that a single enzyme might be involved. However, it is also probable that two enzymes may occur in the preparations: 5'-pyrimidine nucleotidase active around pH 7.00 and a non-specific acid phosphatase active around pH 5.00. Such an assumption may explain why the observed reactivity with UDP was greater than UMP at pH 5.50 and also why the relations were reversed at pH 7.00.

These results show that staphylococcal phosphatase is active on a variety of substrates similar to those of other bacteria, but what possible relationship exists between the occurrence of phosphatase and virulence of staphylococci can hardly be conjectured. In addition to the capacity to hydrolyse phosphate esters, phosphatases possess other functions like, for instance, phosphotransferase activity.

SUMMARY

A study of the properties of phosphatase produced by three strains of *Staphylococcus aureus* was made Staphylococcal phosphatase hydrolysed phenyl phosphates at the highest rates it was more active on 5 pyrimidine nucleotides than on purine nucleotides it hydrolysed sugar phosphates with a reactivity that was higher towards α than that toward β glycerol P Inorganic pyrophosphatase activity was also demonstrable A pH optimum at 5 00–5 50 was found for the hydrolysis of all substrates whereas another optimum at pH 6 80–7 20 was observed with substrates other than the sugar phosphates or the nucleotide triphosphates On the basis of the occurrence of the different pH optima and different rates of hydrolysis of the mono and diphosphate derivatives of uridine at the two pH values the existence of two enzymes is suspected

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The Institute of Tumour Virus Research The University Institute of Medical Microbiology Copenhagen Denmark

INTERACTION BETWEEN ANTIGENICALLY DIFFERENT CELLS

Virus Induced Cytotoxicity by Immune Lymphoid Cells In Vitro

By

CLAUS LUNDSTEDT

Received ?? vi 68

Since 1960 it has been shown repeatedly that lymphoid cells can inflict target cell death *in vitro* Specifically immune lymphoid cells were usually the effector cells (8 17 25) but even normal lymphocytes may be active in this respect provided they are non specifically stimulated or aggregated to the target cells by means of phytohaemagglutinin (PHA) or heterologous antibody (11)

Previous reports of contactual destruction have been concerned with antigenic differences between allo—or xenogeneic cell strains (8 17) or even tumour specific antigens in otherwise syngeneic combinations (5 6) However it would seem to be of interest if a non oncogenic non cytopathogenic virus could induce new or altered antigenicity in cells so that these could be eliminated by contactual destruction by virus immune (or normal) syngeneic cells This might help to elucidate the partly obscure pathogenesis of virus infections and furthermore possibly imply a wider and more general application of the theory of a surveillance mechanism of antigenically and/or structurally altered cells to include virus induced changes (13 15) The present communication describes experiments in which virus immune lymphoid cells cause target cell death in virus infected syngeneic monolayer cultures

MATERIALS AND METHODS

Animals Strictly inbred C H female mice were used The strain has been kept in our laboratory for several years Unsensitized lymphocytic choriomeningitis (LCM) immune and LCM virus-carrier mice were used as lymphoid cell donors Virus-carrier mice were obtained by injecting newborn animals with LCM virus i p By this means a permanent life long tolerance to the virus is induced in the babies (2?) while the mothers nursing their infected offspring develop a strong immunity to reinfection

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The Institute of Tumour Virus Research is supported by grants from the *Einar Willumsen s Memorial Fund* and the *Danish Cancer Society*

The investigations have also been aided by grants from *Statens almindelige Videnskabsfond* and the *D H Foundation*

and high persisting CF antibody titres[1] These mothers were used as immune cell donors The CF antibody content of the serum of the immune mothers was titrated prior to use of the animals to ensure that immunity was present Titres between 32 and 512 were found The blood virus titres in the virus carrier mice were also determined before experimental use and titres between 2 8 and 4 0 LD_{50}/0 03 cc were found

Virus and titration of virus LCM virus of a strain kept in this laboratory by i p passages in C_3H mice was used To obtain a single batch for all experiments L cells in Roux flasks were infected with this LCM virus and after 3 days of culture at 37 C the supernatant medium was harvested and immediately frozen to —70 C at which temperature it was stored until use The titre of this batch of virus remained between 5 8 and 6 3 for at least 7 months

Titration of virus containing material has been described in an earlier report (20) The end points were calculated according to *Kärber*s method (9) and expressed as *log 10* LD_{50}/*0 03 cc*

Complement fixing antibodies The preparation of antigen and the standard technique used for the test have been described previously (23) This method was altered only in that the antigen is now produced from the spleens of infected mice The complement fixation test was performed in Acryl plates the lowest dilution being 1 4 In order to avoid the rather frequent anticomplementary effect of mouse sera some of these were incubated with guinea pig complement for 1 hour at 37 C and thereafter inactivated at 56 C for ½ hour

Cell cultures Earle s strain L C_3H mouse fibroblasts (clone 929) have been used as target cells throughout Stock cultures were grown in Kolle flasks in medium 199 + penicillin and streptomycin supplemented with 10 per cent inactivated calf serum The cells were removed from the flasks by trypsinization and spun and resuspended in the medium mentioned above Two ml of medium containing 100 000 cells were put into Leighton tubes without coverslips and incubated at 37 C for 24 hours Before addition of lymphocytes all cultures were examined microscopically and completely satisfactory cultures were used *i e* cultures with only very few rounded or loose cells and with confluent growth in part of each tube The healthy tubes were then divided in groups of 6–8 and rinsed with 3 × 1 ml of phosphate buffered saline (PBS) —Ca^{++} —Mg before addition of lymphocytes or fresh medium The cultures which were to be infected with virus were mixed with LCM virus while in suspension and then seeded onto their tubes The multiplicity of infection was between 15 and 50

Lymphocytes Normal LCM virus carrier and LCM immune lymphoid cells were obtained by excising the spleens of the animals under sterile precautions The spleens were then pressed through a stainless steel mesh washed 2–3 times in medium and resuspended This medium also contained 10 per cent inactivated calf serum The white cells were then counted in methyl violet in acetic acid and aliquots of 2 × 10^6 white cells in 2 ml of medium were added to each tube Sometimes the red blood cells were haemolysed by suspension in destilled water and the addition after 10 sec of an equal volume of 1 8 per cent NaCl

Cell counting Forty eight and sometimes also 24 hours after the addition of lymphocytes the surviving L-cell nuclei were counted by a method which was a slight modification of that described by *Sanford et al* (19) and *Rosenau & Moon* (18) The cultures were washed 3 times in PBS—Ca —Mg to remove all loose cells and then 1 5 ml of 2 per cent citric acid was added to each tube After incubation for 1 hour at 37 C the tubes were shaken vigorously for a couple of seconds and 0 5 ml of 0 1 per cent crystal violet in 2 per cent citric acid was added The L cell nuclei which were easily distinguished from the lymphocytes were then counted in a standard haemocytometer The actual haemocytometer counts are shown in the tables and figures since only the relations between the different groups are of interest in this study For absolute figures (*i e* number of cells/tube) the multiplication factor is 2281 In other words x̄ = 43 8 in the figures correspond to 100 000 cells The mean ± SF of 6–8 tubes is shown throughout

Fluorescecing antibodies In each experiment Leighton tubes with coverslips were seeded with LCM infected or uninfected cells 24 hours later the cells were fixed in acetone at —20 C for 10 minutes and then incubated with a fluorescein thiocyanate-conjugated C_3H anti LCM hyperimmune serum for 30 minutes at 37 C

1 *Larsen J H* Personal Communication 1967

Hyperimmune serum was obtained and conjugated as described by *Pedersen & Volkert* (16). After washing in PBS and air drying the coverslips were mounted on glass slides with a drop of glycerol containing 10 per cent PBS pH 7.2. The cells were examined in a Leitz microscope equipped for fluorescent studies with an Osram HBO 200 W mercury vapour lamp using 100 × oil immersion lens. The percentage of fluorescing cells was calculated and after 24 hours in culture the average in 15 experiments was 81.8 per cent (range 65 to 98 per cent).

Experimental design. The experiments were carried out as shown in the following scheme

	L cells		Lymphocytes		
Group no	uninfected	LCM infected	Normal	LCM immune	LCM viruscarrier
1	x				
2		x			
3	x		x		
4	x			x	
5		x	x		
6		x		x	
7		x			x

RESULTS

When LCM infected monolayer cultures of L cells are treated with LCM immune spleen cells these cause damage and destruction of the target cells. Table 1 shows a statistically significant ($p < 0.01$) reduction in the number of surviving L cells in six out of 13 experiments 18 hours after the addition of LCM immune lymphoid cells as compared with cultures to which unsensitized lymphoid cells had been added (exp nos 4, 5, 6, 11, 14, 16). In the remaining seven experiments there was no significant difference between the two sets of cultures. It is noteworthy that the number of L cells to survive after 18 hours incubation with unsensitized lymphoid cells was not in any experiment significantly smaller than that in the groups treated with LCM immune lymphoid cells.

On the assumption that no difference between the two experimental groups could be expected the probability of obtaining a result such as that mentioned above (6 experiments displaying significant difference $p < 0.01$ in one direction versus no experiments with significant difference in the other direction) would be $\frac{2}{2^6} = 3.1$ per cent. In other words a result not likely to be obtained by chance.

Within the rates of infection obtained in these experiments (65–98 per cent) no correlation between the percentage of infected L-cells before addition of the lymphoid cells and the eventual result of the experiment could be found. There was likewise no relation between the varying CF titres of the mice used as immune cell donors and the final results. And finally no difference in effect could be found whether or not the red cells of the spleen cell suspensions had been lysed prior to addition to the targets.

Furthermore when the results from each experiment of the groups compared are plotted as shown in Fig. 1 it is obvious that no perma-

TABLE 2

Number of L-Cells in LCM Infected Cultures 24 Hours after Addition of Unsensitized (Group 5) or LCM Immune (Group 6) C_3H Lymphocytes

Exp no	Group	n	$\bar{x}$	SE	p
13	2	6	78.3	5.2	
	5	6	44.2	4.0	< 0.01
	6	6	36.7	3.7	> 0.05
14	2	6	84.3	5.2	
	5	6	39.0	2.3	< 0.01
	6	6	53.3	7.8	0.05 > p > 0.01
15	2	6	174.8	30.2	
	5	6	65.3	11.7	< 0.01
	6	6	95.3	13.6	< 0.01
16	2	6	109.5	14.1	
	5	6	33.0	3.8	< 0.01
	6	6	30.8	3.4	> 0.05

Group 2 = untreated controls

TABLE 3

Number of L Cells in LCM Infected Cultures 48 hours after Addition of Virus Carrier (Group 7) or LCM Immune (Group 6) C_3H Lymphocytes

Exp no	Group	n	$\bar{x}$	SE	p
1	2	6	101.2	16.2	
	7	6	26.3	8.1	< 0.01
	6	6	11.7	1.5	< 0.01
2	2	8	177.9	8.4	
	7	7	30.6	2.0	< 0.01
	6	8	7.9	1.4	< 0.01
5	2	8	201.8	17.6	
	7	8	77.4	4.7	< 0.01
	6	8	47.1	3.6	< 0.01
7	2	8	133.1	9.3	
	7	8	20.8	2.6	< 0.01
	6	8	20.5	1.4	> 0.05
8	2	8	138.9	6.6	
	7	6	42.3	3.2	< 0.01
	6	7	36.6	3.9	> 0.05
9	2	8	176.3	12.1	
	7	8	39.8	5.0	< 0.01
	6	8	[illegible]	3.1	< 0.01
10	2	8	211.9	9.8	
	7	8	30.0	3.5	< 0.01
	6	8	47.9	4.5	< 0.01
11	2	8	244.1	9.8	
	7	8	144.8	9.3	< 0.01
	6	8	48.3	3.9	< 0.01

Group 2 = untreated controls

significant reduction ($p < 0.01$) in the number of surviving L cells in the groups treated with LCM immune cells as compared with the groups treated with virus carrier cells In two experiments (nos 7 and 8) there were only small differences whilst in the remaining two (nos 9 and 10) there were significantly fewer surviving L-cells after 48 hours incubation in the groups treated with virus carrier cells

The series of experiments in which uninfected L-cells were employed but the experimental conditions otherwise were the same revealed a quite different pattern After 48 hours of incubation with either LCM immune or unsensitized lymphoid cells no significant differences in the number of surviving L-cells were found in six of the eight experiments (Table 4 nos 14–19) In the remaining two however there were significant differences one having the smallest number of L-cells in the group treated with immune cells (no 13) and the second the smallest number in the group treated with unsensitized cells (no 20) Thus no clear cut tendency in favour of either of the treatments could be found

In similar experiments which were already counted after 24 hours

TABLE 4

Number of L Cells in Uninfected Cultures 48 Hours after Addition of Unsensitized (Group 3) or LCM Immune (Group 4) C_3H Lymphocytes

Exp no	Group	n	x	SE	p
13	1	6	170.8	6.7	
	3	6	35.7	5.2	< 0.01
	4	6	21.2	2.4	< 0.01
14	1	6	209.3	10.0	
	3	6	45.5	4.8	< 0.01
	4	6	57.5	11.6	> 0.0
15	1	6	350.5	57.8	
	3	6	55.0	8.9	< 0.01
	4	6	62.8	11.5	> 0.05
16	1	6	307.0	24.2	
	3	6	40.8	4.5	< 0.01
	4	6	47.2	8.5	> 0.05
17	1	6	206.0	10.1	
	3	6	90.3	7.4	< 0.01
	4	6	98.3	4.6	> 0.05
18	1	6	211.7	9.0	
	3	6	114.5	3.6	< 0.01
	4	6	118.7	7.8	> 0.05
19	1	6	284.8	13.9	
		6	147.0	12.3	< 0.01
	4	6	133.2	8.9	> 0.05
20		6	180.8	8.9	
	3	6	40.8	3.5	< 0.01
	4	6	74.7	4.7	< 0.01

Group 1 = untreated controls

TABLE 5

Number of L Cells in Uninfected Cultures 24 Hours after Addition of Unsensitized (Group 3) or LCM Immune (Group 4) C_3H Lymphocytes

Exp no	Group	n	$\bar{x}$	SE	p
13	1	6	113.5	6.4	
	3	6	43.5	2.5	< 0.01
	4	6	30.0	2.4	ca 0.01
14	1	6	110.2	4.0	
	3	6	51.5	4.7	< 0.01
	4	6	64.5	5.8	ca 0.05
15	1	6	175.0	17.4	
	3	6	94.0	12.4	< 0.01
	4	6	113.7	8.5	> 0.05
16	1	6	103.8	11.7	
	3	6	45.7	3.4	< 0.01
	4	6	47.8	4.7	> 0.05

Group 1 = untreated controls

of exposure to lymphoid cells there were only minor differences between the groups treated with the two types of lymphoid cells (immune or unsensitized Table 5) Here again the number of experiments evaluated at this time (24 hours) is too small to give anything but an indication of the time course

An interesting finding was the dramatic reduction in the number of surviving L-cells in the treated as compared with the untreated groups regardless of whether the lymphocytes used were unsensitized immune or virus carrier cells This reduction was seen in all experiments but one (Table 1 no 4) and indeed of all the untreated groups this one showed the smallest number of L-cells indicating a poorer growth of L-cells *in this experiment Furthermore this reduction* was far greater than that seen as a result of addition of immune cells instead of unsensitized cells to LCM infected L-cells Indeed statistically significant differences between the untreated groups and those treated with the lymphoid cells were already seen after 24 hours of incubation in all experiments (Tables 2 and 3)

The effect of the LCM infection as such on the multiplication of the L-cells was also studied As was to be expected the multiplication rate was slightly lower when the cells were infected than when they were uninfected (Fig 2) This evaluation would have been of the greatest importance if no differences between the effects of unsensitized and immune lymphoid cells on infected L-cells had been found as this could mean either that neither of the two types of cells was effective or that both of them could inflict target cell destruction to an equal degree This could be judged by comparing the effects of the two types of cells on uninfected and LCM infected L-cells respectively due attention being paid to the effect of the LCM infection as such

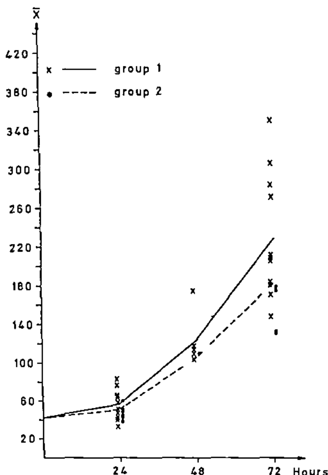


Fig 9

Numl er of uninfected (group 1) an l LCM infected (group 2) L cells after 24 48 an l 72 hours in culture x = Mean of 6-8 tubes

LCM virus titrations on the media of the different experimental groups were performed in some cases at the end of the experimental periods The virus titres were always about 4 0 irrespective of the type of lymphoid cells added or even of whether or not lymphoid cells w r added Thus in these experiments the virus contents of the medi did not seem to be rel t d to the d gree of l struction of cells in th l tures However at the e d f 18 hour period some cells wer s left unharm d th ble st capable of produ us

l SS N

It has b nstr t d t at LC infection of l us renders these s n nta tu truct by LCM imm syngeneic

lymphoid cells and b) that an incompatibility between the L-cells and the inbred C_3H lymphoid cells used by the author exists

The demonstration that a virus (LCM) which in itself is neither cyto pathogenic nor oncogenic can make L-cells sensible to a cytocidal complement independent action of syngeneic lymphocytes extends the implications of the contactual destruction phenomenon This reaction has hitherto been used in the study of auto immune diseases (1) the homograft reaction (3) tumour immunity (5 6) and the allogeneic inhibition phenomenon (4)

It has not been possible in the present study to prove whether destruction or merely varying degrees of inhibition of mitosis has occurred but the finding of mitosis figures even in severely damaged cultures and of partly lysed cells by microscopical examination strongly indicates the real possibility of destruction

The reason for the lack of significant differences between the experimental and control groups in seven of the 13 experiments (whilst there was certainly no inversely significant differences) has not been elucidated *The lack of correlation from one experiment to the next* was in all events not due to the varying contents of CF antibodies in the mice used Cells from mice with low or high contents of CF antibodies were not distinguishable in their effect on the target cells

However the present technique is very sensitive to exogenous variations such as varying growth conditions caused by different brands of Pyrex tubes But it is more probable that the reason is the relatively small lymphocyte/target cell ratio used (20 1 when calculated at the time of seeding) If the reports of *Wilson* (26) and *Miller et al* (10) viz that only 1 2 per cent of the lymphocytes in a sensitized population are immunologically active against the antigen in question and that only one lymphocyte deals with one target cell are accepted then for the present investigation this would imply that one or two lymphocytes were available for every five target cells This would not be sufficient for an optimal effect and it could be expected that a higher percentage of significant results would have resulted from increased lymphocyte/target cell ratios A lymphocyte dose dependence has already been demonstrated in other investigations using contactual destruction (2 7 26)

The fact that the fall in surviving L cells in the LCM infected tubes is not an effect of the immune lymphocytes alone is seen from the uninfected controls in which no difference between the effect of normal and immune cells could be found Only when the LCM antigen is present *in the L cells can the effect of the immune lymphocytes be demonstrated*

Furthermore the four experiments (nos 13 16) in which all groups were processed simultaneously indicate that the normal lymphocyte caused no damage to the L cells because of LCM infection of the latter as no constant difference between this group (group 5) and the uninfected groups (groups 3 and 4) with lymphocytes added could be

found even not when the effect of the LCM infection itself on the L cells is considered

It is an interesting feature that the use of virus carrier lymphocytes as controls instead of unsensitized lymphocytes did not alter the observed fall in the treated as compared with the untreated groups On the contrary there is an indication that these lymphocytes in their effect were rather comparable with the immune lymphocytes This might mean that in the virus carrier population there might also exist immune cells which could exaggerate a visible effect *in vitro* but not *in vivo* However such cells have not as yet been demonstrated with certainty in virus carrier mice Results however are too few and so far the evidence cannot be considered conclusive further experiments are needed

The dramatic fall in the number of surviving L cells after addition of all types of lymphocytes (as was even seen in 7 experiments in which normal or L-cell immune Swiss mice lymphocytes were added to uninfected L-cells) was an unexpected finding in this series of experiments It was anticipated that the L-cells which by now have lived for more than 20 years in laboratory cultures could hardly be expected to be strictly syngeneic to any strain of inbred C_3H mice Mutations in one or both parts might have occurred On the other hand possible antigenic discrepancies were not thought to be a major obstacle in the present system For this reason and because of difficulties in the precise quantification of primary explanted mouse embryo fibroblasts the L cells were chosen as targets The results are difficult to explain but several possibilities exist Firstly substrate competition might be responsible for the non specific fall in the groups of L-cells treated with lymphocytes However this is not very likely since other authors have used the same number of cells even with half the amount of medium and it has been shown that competition for substrate occurs only when more than 10×10^6 lymphocytes per culture are used[1] Secondly the lymphocyte suspensions might be toxic but haemolysis of the red blood cells + three times washing of the now pure white cell suspension did not influence the results although this procedure does not exclude the possibility of toxic factors Thirdly the batch of calf serum used might be responsible as it was shown that it contained a natural agglutination to mouse red cells in a titre of 1 8 as has also been described by *Moller* (12) It has been demonstrated that allogeneic inhibition *in vitro* [illegible] effected not only by immune but al[illegible] by normal lymphoid cells although in the latter case only if uns[illegible] aggregation to the target [illegible] brought about by substances [illegible] HA or heterologous antisera [illegible] light of this concep[illegible] on [illegible] the [illegible] lymp[illegible] non specifically [illegible]ted by the [illegible] calf serum [illegible]tigenically and [illegible]turally

[1] R [illegible] Commu[illegible] 7

different L cells and thereby showed allogeneic inhibition. The immune status of the lymphocytes to an antigen (LCM) which is irrelevant in this respect would make no difference. This mechanism could account for at least some of the reduction observed in lymphocyte treated groups as compared with untreated groups. It would be natural to omit calf serum in a new series of experiments and preliminary results from such experiments indicate that the fall in number of target cells is not as great as that observed in the present investigations; in some experiments it is not demonstrable. Despite this the effect of LCM immune lymphoid cells on infected L cells would seem not to be altered.

The present observation is of importance since it shows that not all inbred strains of C_3H mice can be regarded as syngeneic with L-cells and this probably explains some of the discrepancies when results from different laboratories concerning the outcome of the addition of C_3H lymphoid cells to L cells are compared. This also calls for circumspection when other experimental results involving the use of both L-cells and C_3H mice are compared.

The finding that the C_3H—L cell incompatibility could be demonstrated by non immune lymphoid cells while the LCM induced new antigens were visualized only by LCM immune lymphoid cells might be a question of the concentration of antigenic surface determinants. It has been shown that this concentration is of importance for contactual destruction (7). Thus it could be speculated that the LCM infection contributes so few antigenic determinants to the cell that they could be demonstrated only by LCM immune cells, whereas the incompatibility between the L cells and the C_3H cells used involves a greater concentration of different antigenic determinants resulting in a demonstration of the incompatibility also by unsensitized cells.

Neither interferon nor humoral antibodies seem to play a major or causal role in the elimination of LCM virus from infected animals (21–24). It might therefore be speculated that the contactual destruction phenomenon could represent a virus eliminating function in vivo analogous to the way in which tumour cells by some authors are considered to be eliminated (1[illegible]). Such a mechanism might supplement the partly incomplete knowledge of virus elimination, especially if one imagines a certain organ specificity parallel to the varying organotropism of different viruses. The absence of any difference in virus titres in the media of the experimental groups does not necessarily exclude such a possibility since a number of intact cells were always present in the cultures at the end of the experimental period.

SUMMARY

Virus induced altered antigenicity in cultured cells was investigated using the contactual destruction phenomenon. L cells were infected with a non-cytopathogenic non oncogenic virus (LCM). Addition of

LCM immune C_3H spleen cells led to destruction of the monolayer target cultures after 48 hours in about half of the experiments Un sensitized cells caused no damage which could be attributed to the LCM infection while virus carrier cells (tolerant) had a destructive effect in a few experiments The latter observation might indicate the presence of immune cells in the tolerant population

Another interesting finding in these experiments was that both un infected and LCM infected L-cells were damaged to a considerable degree by all types of C_3H lymphoid cells The reason was thought to be the use of calf serum containing a natural agglutinin to mouse red cells in the culture media This serum would cause aggregation to the L cells of the lymphoid cells By this means it was possible to demon strate an antigenic difference between the L cells and the C_3H cells used in the present study

The present study gives wider implications to the contactual de struction phenomenon The possible role of this phenomenon is a virus eliminating mechanism is discussed

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The Mycology Laboratory (Head Dr Per Holm) Statens Seruminstitut and The Department of Restorative Dentistry and Dental Pathology (Head Professor odont dr h c Johs Juul Holst) The Royal Dental College Copenhagen Denmark

ALTERATION IN VIRULENCE IN STRAINS OF *CRYPTOCOCCUS NEOFORMANS* RESISTANT TO AMPHOTERICIN B AND POLYMYXIN B

With a Contribution to the Discussion Regarding the Relationship between Encapsulation and Virulence of Cryptococcus neoformans

By

Jessie Bodenhoff

Received 1 xii 68

Obvious changes in virulence associated with the development of resistance have been observed in *Mycobacterium tuberculosis* (*Middlebrook & Cohn* 1953)

Similar changes have also been observed in the yeasts *Lones & Peacock* (1959) found that two strains of *Candida albicans* in which the resistance to amphotericin B was increased by 13 and 29 times respectively showed a significantly lower virulence in the mouse than did the parent strains

Hebeka & Solotorovsky (1962) found that candidin resistant strains of *Candida albicans* were less virulent to the mouse than the parent strains The same authors observed 3 years later (1965) that strains of *Candida albicans* with increased resistance to amphotericin B were similarly less virulent than the parent strains

In an earlier publication (*Bodenhoff* 1968) it has been demonstrated that it is possible to induce a considerable resistance to amphotericin B and polymyxin B in *Cryptococcus neoformans*

The present paper contains a report of some experiments designed to discover whether increased resistance in *Cryptococcus neoformans* is associated with decreased virulence for mice

MATERIAL AND METHODS

The experiments were primar[illegible] i ned as ordina[illegible] ns in the mouse *i e equal groups* [illegible] *ls were inf* [illegible] *train* or the derived strain observ[illegible] f certain numb r [illegible] Jead to living animals in each gr[illegible] tabli hed

In addition in each g[illegible] f "extr[illegible] Such mice had not alre dy die[illegible] nfecti n [illegible] intervals and

survived for 80 days whilst *only three of the ten inoculated with the* parent strain survived.

TABLE 2
Experiment 2
Virulence in Cryptococcus neoformans
Relation between Virulence in a Sensitive Strain and a Derived Polymyxin B Resistant Strain

Strain No	Exposed to antimycotic	Number of transfers	Degree of resistance	Infecting dose	Survivors after 80 days
M 14-372		11		6.5 × 10⁵	3/10
M 14-372	Polymyxin B	11	10 ×	7.5 × 10⁵	10/10

It is furthermore noteworthy that the results of infection with the parent strain are as uniform as could be expected with three survivors out of 10 in one group and two out of nine in the second.

The results of the cultures from the killed and dead animals which are recorded in Tables 3, 4, 5 and 6 give further details of the course of the infections in the four groups.

Tables 3 and 5 contain the infection experiments using the original sensitive (= parent) strain (OR). It is apparent from the tables that the first death among mice in these groups occurred as early as five days after infection and that the majority of the remainder of the animals died within three weeks of infection.

It may be seen from the results of the cultures from the killed animals numbered with Roman numerals that by the fifth day there is already massive growth of cryptococcus from all organs (liver, spleen, kidneys, *lungs and brain). Similar massive infection was found in the* animals killed on the 10th, 20th and 30th days and also in all the animals which died during the experiment.

Some of the animals survived for more than 80 days. Among these mouse OR 10 died on the 82nd day (Table 3); numerous cryptococci were found in all organs. Mice numbers OR 13, OR 16 and OR 18 *(Table 5) and mouse OR 8 (Table 3) were killed on the 84th, 88th,* 88th and 160th days respectively. From the cultures it would appear that mouse OR 13 was in the process of overcoming the infection, mouse OR 8 was perhaps overcoming it, but in mice OR 16 and OR 18 there were such large amounts of cryptococcus in the organs, in particular in the brain, that it is doubtful whether the course of the infection was decided in these animals. *Apparently it would have been preferable if* all the animals had been observed for 160 days.

The course *of the infection in the* animals infected with amphotericin B resistant cryptococcal cells (AB) was quite different. As shown in Table 4 there were no early deaths among these mice but two of the

mice (AB 4 and AB 9) were moribund on the 57th day. These animals were obviously extremely ill: both had ulceration about the nose, pustules and ulcers on the auricular cartilage, tumour-like masses corresponding to the segments of the tail and on the lower fore and hind legs there were panaritium-like bullous lesions. Both these animals were killed. Culture revealed massive growth in all organs. In the account of the results of the virulence experiments these animals are counted as "dead".

Mouse AB 2, which died on the 47th day, has been excluded from the experiment, as culture from the organs revealed plentiful growth of Escherichia coli and scanty growth of cryptococcus. This animal has not been included in the virulence results shown in Table 1, which therefore shows that two animals out of nine died within 80 days.

In the cultures from the animals killed on the 5th, 10th, 20th, 30th, 40th and 50th days after infection it was also found (see Table 4) that there was scanty growth from the organs of the animals which were killed soon after infection, and that the longer the animal had lived after the infection the greater the number of colonies of cryptococci which were cultured from the organs. There was massive growth of cryptococci from all the organs, including the brain, in the animal which was killed on the 50th day.

It would appear that either the amphotericin B resistant cryptococci do not reproduce themselves in the animal during the period immediately after infection or that they only reproduce themselves slowly.

From the studies of the six animals which survived for 160 days it is also apparent that the course of the infection after the 50th day presumably follows one of two courses: either the animal recovers (mice AB 5, 6 and 7) or else it develops a chronic cryptococcal infection (mice AB 1 and 8).

Mouse AB 10, which died on the 139th day with numerous cryptococci in all organs, is a typical example of a mouse with a chronic cryptococcosis. Mouse AB 1 revealed, in addition to the massive infection of the brain, widespread cutaneous signs of cryptococcosis. In mouse AB 8 there was growth of numerous colonies of cryptococci in samples from the kidneys and lungs and a large number from the brain. In mouse AB 3 there was a scanty growth of cryptococci in the brain but no growth in any other organ.

Table 6 shows the results of culture experiments on the animals which were infected with the polymyxin B resistant strain (PB). The course in these animals was quite different both from that found in animals infected with the parent strain (Tables 3 and 5) and also from those infected with the amphotericin B resistant strain (Table 4). On culture of samples taken from the mouse killed on the fifth day after infection there was growth of large numbers of cryptococcal colonies from the liver and spleen and some or a few from the lungs, kidneys, blood and brain. In the mice killed on the 10th, 20th and 30th

survived for 80 days whilst only three of the ten inoculated with the parent strain survived

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3
of Cryptococcus neoformans *M 14–37?*
approx 5.? × 10⁵

from organs Spleen	Kidneys	Lung	Blood	Brain	Nose fingers tail
++++	++++	+++++	+	++++	
++++	+++++	+++++	++	+++++	
+++++	+++	+++++	+	+++++	
+++++	+++++	+++++	+	+++++	
+++++	+++++	+++++	++	+++++	
+++++	+++++	+++++	(+)	+++++	
+++++	+++++	+++++	+++	+++++	
+++++	+++++	+++++	++	+++++	
+++++	+++++	+++++	(+)	+++++	
+++++	+++++	+++++	+	+++++	
+++++	++++	+++++	0	+++++	
+++++	++++	+++++	++	+++++	
+++++	+++++	+++++	0	+++++	
(+)	0	++	0	+++++	
+++++	+++++	+++++	0	++	
+++++	+++++	+++++	0	+++++	

++ 10–50 colonies
+++ 50–100 colonies
++++ several hundred colonies
+++++ many hundred colonies (> confluent growth)

4
Strain of Cryptococcus neoformans *M 14–37?*
approx 7.5 × 10⁵

from organs Spleen	Kidneys	Lungs	Blood	Brain	Nose fingers tail
++	(+)	+++	0	(+)	
++	+	+	0	+	
+++	+	++	0	++++	
(+)	0	0	0	+++++	
+++	+	++	0	+++++	
++++	+++	++++	++	+++++	+++++
(+)	0	0	0	+++++	+++++
	++	++	(+	++	
0	0	0	0	++	
++++	+++++	+	++	+++++	+-
0	0	0	0	0	
0	0	0	0	0	
0	0	0	0	0	
+	- +++	++++	( )	+++	
+++++	- +	-+++	-)	+++++	-
+ +++	--++	----+	+-+-	+++++	

TABLE

Infection Experiments Using the Parent Strai
Infection dos

Experiment 5/7 1966			Mesentery	Culture Peritoneum
Mouse OR VII	killed	5th day	+++	(+)
Mouse OR VIII	dead	10th day	++++	++
Mouse OR IX	killed	10th day	+++	+
Mouse OR X	dead	7th day	++++	++++
Mouse OR XI	dead	7th day	++++	++++
Mouse OR XII	killed	90th day	++++	0
Mouse OR 11	dead	12th day	++	+
Mouse OR 12	dead	19th day	++	+
Mouse OR 13	killed	85th day	0	0
Mouse OR 14	dead	15th day	++++	+++
Mouse OR 1	dead	14th day	+++	+
Mouse OR 16	killed	88th day	0	0
Mouse OR 17	dead	27th day	0	0
Mouse OR 18	killed	88th day	0	0
Mouse OR 19	dead	5th day	++++	++
Mouse OR 20	dead	9th day	++++	++

Key to Registration (Tables 3 C)

	no culture experiment
0	no colonies
(+)	few colonies (> 1 2 or 3)
+	4 9 colonies

TABLE

Infection Experiments Using the Polymyxin B Resistant
Infection dose

Experiment 5/7 1966			Mesentery	Culture Peritoneum
Mouse PB XIII	killed	5th day	(+)	0
Mouse PB XIV	killed	10th day	0	(+)
Mouse PB XV	killed	20th day	0	0
Mouse PB XVI	killed	22nd day	0	0
Mouse PB XVII	killed	30th da	0	0
Mouse PB XVIII	killed	40th day	0	0
Mouse PB 21	killed	80th day	0	0
Mouse PB 22	killed	80th da	0	0
Mouse PB 23	killed	80th day	0	0
Mouse PB 24	killed	80th da	0	0
Mouse PB 25	killed	80th day	0	0
Mouse PB 26	killed	90th day	0	0
Mouse PB 27	killed	90th day	0	0
Mouse PB 28	killed	90th day	0	0
Mouse PB 29	killed	90th day	0	0
Mouse PB 30	killed	90th day	0	0

5
of Cryptococcus neoformans *M 14–372*
approx 6.5 × 10⁵

from organs Liver	Spleen	Kidneys	Lungs	Blood	Brain
+++++	+++++	+++++	+++++	++	+++++
+++++	+++++	+++++	+++++	++++	+++++
++++	++++	+++++	+++++	(+)	+++++
+++++	+++++	+++++	+++++	++	+++++
+++++	+++++	+++++	+++++	++	+++++
+++++	+++++	+++++	+++++	+	+++++
+++++	+++++	+++++	+++++	+	+++++
+++++	+++++	+++++	+++++	+	+++++
0	+	0	++++	0	0
+++++	+++++	+++++	+++++	++++	+++++
+++++	+++++	+++++	+++++	+	+++++
0	++	++++	++++	0	+++++
++++	+++++	+++++	+++++	(+)	+++++
(+)	+++	+++++	++	0	+++
+++++	+++++	+++++	+++++	+	++++
+++++	+++++	+++++	+++++	++	++++

++ 10–50 colonies
+++ 50–100 colonies
++++ several hundred colonies
+++++ many hundred colonies (≈ confluent growth)

6
Strain of Cryptococcus neoformans *M 14–372*
approx 7.5 × 10⁵

from organs Liver	Spleen	Kidneys	Lungs	Blood
++++	++++	++	+++	(+)
+++	+++	(+)	0	0
+	+	0	0	0
+	+	0	0	0
+	0	(+)	0	(+)
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0

intraperitoneal
ised in 1

days after infection there were fewer and fewer colonies of cryptococci the longer the interval between the infection and the sacrifice of the animal On culture of tissues from the animal which was killed on the 40th day and also those from the virulence determination experiments there was no evidence of cryptococcal infection At this time none of the animals all of whom most presumably have had cryptococci in the organs during the first days after infection (vide the animal killed on the fifth day) showed any evidence of cryptococcal infection Investigation of the killed animals has thus in every case completely confirmed the impression that the *polymyxin B resistant strain has completely lost* its virulence

This observation from experiment 2 that the polymyxin B resistant strain had completely lost its virulence was considered extraordinary *and some experiments were therefore carried out to search for an ex*planation of the finding

It is conceivable that the reason for the survival of the infected mice was that the cells of the polymyxin B resistant strain were as has been *demonstrated previously (Bodenhoff 1968) partially dependent on* polymyxin B and that it was therefore easy for the natural resistance mechanism in the mouse to combat the injected cryptococci

Experiment 2 was therefore repeated with the following modifi*cations*

(1) during the experimental periods the mice were treated with intramuscular injections of polymyxin B (the dosage was 0 04 mg polymyxin B per mouse at first daily later at intervals of 2–4 days) and
(2) culture from the organs of the killed animals was carried out on Sabouraud s agar to which polymyxin had been added in a concentration of 25 μg/ml

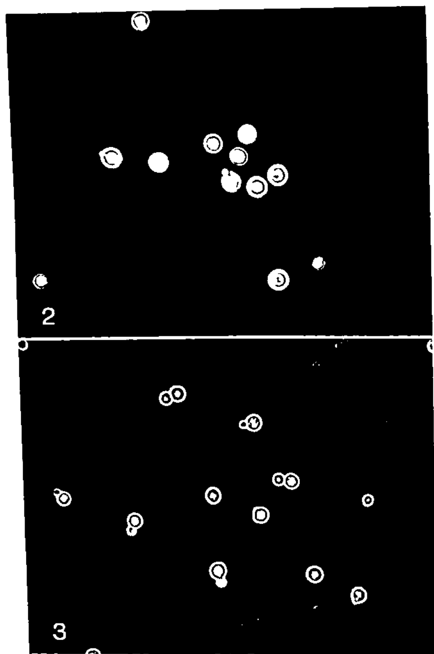
The results of the modified experiment 2 were exactly the same as those of the original experiment

In order to investigate what happens to the cryptococcal cells after they are injected into the mouse the following experiment was set up

Suspensions of both the original sensitive cryptococcal cells and of the polymyxin B resistant cells were made On microscopy of India ink mounts of the two suspensions the cryptococci in both were found to be very small having very thin capsules which was perhaps slightly more obvious in the sensitive cryptococcal cells than in the polymyxin resistant cryptococcal cells (Figs 2 and 3)

Figs 2–7

All photographs enlarged to the same degree (× 900)



Figs 2-3

Fig 2 Suspension of original sensitive cryptococcal cells used in intraperitoneal injection

Fig 3 Suspension of polymyxin B resistant cryptococcal cells used in intraperitoneal injection

Two groups of mice were then given intraperitoneal injections of the two suspensions (dose approx 10^6 cryptococcal cells) Twenty four 48 and 72 hours after the infection a small amount of peritoneal fluid was removed from the animals by means of a capillary Pasteur pipette The exudate was mixed with India ink and examined microscopically This revealed that the development of the cryptococcal cells was different in the two groups of animals In the peritoneal exudate from the animals infected with sensitive virulent cryptococcal cells it was seen that these showed an obvious increase in size there was an immense increase in the size of the capsule to such a degree that it was possible to find cells with capsules which were the same size as or even larger than those found in the cerebrospinal fluid in patients with cryptococcosis There was moreover evidence of very vigorous reproduction which apparently took place at a high rate (Fig 6)

The polymyxin B resistant cryptococcal cells showed some increase in size and their capsules were also slightly thicker than those in the suspension which had been used for the infection However the capsules were obviously much thinner (Fig 7) than those in the sensitive cryptococcal cells obtained from the peritoneal fluid in the other group of mice Occasionally cryptococcal cells which were apparently being phagocytosed were seen (Fig 5)

It would thus seem as though there is a change in the ability of the cells to produce capsules of normal qualities in the cryptococcal cells resistant to polymyxin B

DISCUSSION

The course of the cryptococcal infection in the mouse is well known from the literature (*Littman & Zimmerman* 1956) The course of the infection in those mice which in the present study were infected with the parent strain is in accordance with this—when allowances are made for the relatively small infective dosage The course in those mice which were infected with amphotericin B resistant and polymyxin B resistant cryptococcal cells was so obviously divergent from this that no explanation of the difference in the resistance of the animals which have been used can be found

There is thus a great deal of evidence which would suggest that the induction of resistance to antimycotic agents leads to a loss of virulence in cryptococcal cells corresponding to that which as mentioned in the introduction, has been observed in Candida albicans by *Jones & Peacock* (1959) and *Hebeka & Solotorovsky* (1962)

The difference between the loss of virulence in the amphotericin B resistant and the polymyxin B resistant strains is extraordinary

In the amphotericin B resistant cryptococcal cells the virulence is obviously reduced but still present This may perhaps be explained the following manner It has been demonstrated that resistant cryp

tococcal cells have a reduced rate of growth (*Bodenhoff* 1968). It is quite conceivable that this would give the host organism a greater opportunity to combat the infection or at all events to delay its course. Moreover it is known from the study quoted above that amphotericin B resistant cryptococcal cells rapidly lose their resistance when they are confronted with a substrate which contains no antimycotic. On the assumption that a reduction of resistance is immediately followed by an increase in virulence the infection in the mice infected with the amphotericin B resistant cells may perhaps be considered to be a mixed infection of resistant cells of low virulence and a small number of sensitive virulent cells which gradually spread through the mouse until it succumbs.

The total loss of virulence in the polymyxin B resistant cells cannot however be explained in this way. In this case it must be assumed that the polymyxin B resistant cells are altered in such a way that the mouse's body is capable of combating the micro organism.

In this connection the observation of the change in the ability of the polymyxin B resistant cryptococcal cells to develop capsules of the usual size *in vivo* is of considerable interest particularly when the simultaneous alteration in the composition and properties of the capsule which is formed is taken into consideration.

For a number of years it has been discussed whether there is any correlation between the capsule and virulence in *Cryptococcus neoformans*. Thus *Drouhet et al* (1950) consider that the virulence in yeasts is directly dependent on the size of the capsule. *Kao & Schwartz* (1957), *Littman & Tsubura* (1959) and *Hasenclever & Mitchell* (1960) have been unable to confirm this. *Kase & Metzger* (1962) isolated a small capsule variant of *Cryptococcus neoformans* which was more virulent than the large capsule parent strain. *Bulmer et al* (1967) induced a number of uncapsulated mutants and found that these were nonpathogenic to the mouse whilst the capsule forming parent strain was lethal to the mouse. They considered the capsule as a virulence factor.

The experiments reported in this paper must be considered to support the assumption that the capsule in *Cryptococcus neoformans* is a virulence factor as changes in the capsule are accompanied by a loss of virulence.

SUMMARY

An amphotericin B resistant and a polymyxin B resistant strain induced from the same parent strain of *Cryptococcus neoformans* have been investigated for virulence in the mouse. In both there was a marked alteration. The amphotericin B resistant strain was obviously less virulent than the parent strain. The polymyxin B resistant strain showed a complete loss of virulence.

In the polymyxin B resistant strain the ability to form capsules *in vivo* was found to be obviously changed. It is natural to connect this

change in the ability to produce capsules of normal qualities with the loss of virulence

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Acta path microbiol scandinav 75 169-176 1969

The Mycology Laboratory (Head Dr Per Holm) Statens Seruminstitut and The Department of Restorative Dentistry and Dental Pathology (Head Professor odont dr h c Johs Juul Holst) The Royal Dental College Copenhagen Denmark

CHRONIC CRYPTOCOCCOSIS IN THE MOUSE

By

JESSIE BODENHOFF

Received 13 vii 68

Intracerebral infection with *Cryptococcus neoformans* in white mice leads to an acute illness which is usually lethal A few days after infection the mouse becomes quiescent it crouches in one corner of the cage and its coat roughens On about the fifth day it develops cerebral signs and staggers round in circles Often the occipital region of the skull bulges prominently Microscopy of the cerebral tissue at this stage reveals large numbers of cryptococci The infection generally spreads to other organs where it is similarly possible to demonstrate the yeasts both by microscopy and on culture (*Littman & Zimmerman* 1956 *Riewerts Eriksen & Erichsen* 1962)

Intravenous *Cryptococcus neoformans* infection in the mouse usually gives rise to an acute septic infection The animal becomes ill with signs of generalized infection The illness is almost invariably fatal Large numbers of cryptococci can be demonstrated on culture of the organs including the brain from the dead animals and occasionally from the blood

The various strains of *Cryptococcus neoformans* do not all exhibit the same virulence (*Benham* 1950 *Hasenclever & Mitchell* 1960) Some strains are particularly virulent and when given intravenously in sufficiently large doses they kill the animal within two days (*Holm* 1968) Other strains take longer to kill the mouse but the majority are fatal within 14-16 days

However there are also strains of *Cryptococcus neoformans* which possess only very low virulence (*Benham* 1950 *Hasenclever & Mitchell* 1960)

In the course of a series of experiments (*Bodenhoff* 1969) using an artificially derived low virulent strain of *Cryptococcus neoformans* partly before and partly after passage in a mouse 14 out of 32 mice which had been infected intravenously developed a chronic illness with

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The author expresses her thanks to *T y Møll r* Associate Professor Department of Pathology The Royal Veterinary and Agricultural College Copenhagen Denmark for his assistance in the histological stud

a strange and interesting clinical course which has not hitherto been described It would therefore seem justified to give a short description of some of the findings in mice with chronic cryptococcosis

MATERIAL AND METHODS

The experiments have been carried out with an amphotericin B resistant strain derived from *Cryptococcus neoformans* strain M 14-372 (originally isolated from pigeon droppings by *Riewerts Eriksen & Erichsen* 1962) partly before and partly after passage in a mouse The animals used in the experiments were infected with approx 10^6 cryptococci per mouse For further details of the induction of resistance to amphotericin B in the strain used the assessment of the virulence of this strain and the mycological methods employed in the investigation of the mice reference is made to two earlier reports (*Bodenhoff* 1968 and 1969)

In a few cases histological investigations of organs from the mice have been carried out The tissue was fixed in 10 per cent neutral formalin The specimens were embedded *en bloc* in paraffin and cut into sections 4 µ thick The sections were stained with haematoxylin eosin and the periodic acid Schiff's reagent (PAS)

CLINICAL PICTURE

The illness in the mice which have been observed in this study may be divided into three stages

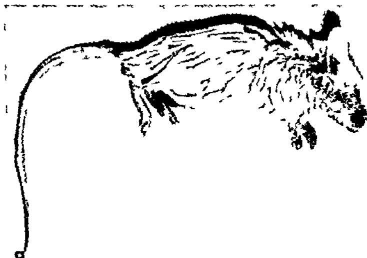
1 A primary stage (from infection to about 5th post infection week) in which the mouse is apparently unaffected

2 A secondary stage (from 5th post infection week) in which the mouse appears distressed When removed from its cage it makes no attempt to run away there is pronounced photophobia the tail stands straight up in the air and it is possible to observe barely visible cutaneous and subcutaneous nodules (see below) Ulcers may develop on the nose and/or shoulder region (Figs 1 a and b)

3 A tertiary stage (after about 7th post infection week) with exacerbation of the processes already observed the mouse eats but its general condition is affected the pulse rate is increased the coat roughens the animal scratches the ulcerations on its nose The extent of the ulcerations increases they become confluent and form prominent granulomas The nodules on the tail increase in size There is bulla formation on the lower fore and hind legs Later in the course of the illness the nodular growth takes on the character of tumour like masses At the same time it is possible to observe cutaneous polygonal discharging ulcers about 1 2 × 1 2 mm in size without hair encrusted occasionally indurated on the area of the sinus hairs on the nose and the shoulder region As the processes continue the ulcers coalesce and in the sinus hair region they grow rapidly to form protruding ulcerating granulomatous lesions which may extend up to the eye involving the upper and lower lips the mucous membranes in the anterior part of the mouth and the anterior nare In addition to these changes it is possible at this stage to see one or more polygonal 5 × 5 mm pustule at the base of the ears in the angle between the lateral aspect of the auricular cartilage and the skin of the head in the infra temporal region These pustules may perforate to the interior of the auricular cartilage but are largest on the external aspect there may also be ulcers in this region Finally there may be panaritium like bullous lesions on the fore and hind legs (Fig 1 c and d)

MYCOLOGICAL EXAMINATION

On puncture of the tumour like lesions on the tails of the animals and of the auricular and digital pustules the platinum needle entered cyst like cavities containing a greyish transparent gelatinous exudate Microscopy of Indian ink preparations of this exudate revealed that it was entirely composed of round to ovoid cells resembling yeast cells





Figs 1 a b
Se ndary stage of chronic cryptococcosis in the mouse Early caudal nodular growths Ulceration on the nose

surrounded by thick capsules Cultures from the exudate grew yeasts the properties of which were the same as those of the yeasts used for the infection of the animals

There was similarly growth of *Cryptococcus neoformans* on culture of samples from the ulceration in the shoulder region and from ulcers and/or granulation tissue from the nose





Figs 1 c d

Tertiary stage of chronic cryptococcosis in the mouse Aggravation of caudal nodular growths Granuloma on the nose Pustule formation on external and internal surfaces of auricular cartilage Panaritium like bullous lesion on the lower fore and hind legs

At the conclusion of the experiments cultures were made from the organs of the animals These revealed growth of cryptococci from the brain and from the majority of the organs occasionally also from the blood of the animals



Figs ? a b

Section from n e cf mouse H.E. stain howing variations in size and shape of cryptococci yeasts lying partly intercellularly partly intracellularly in cytoplasm of large epitheli id cell like cells × 900

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Acta path microbiol scandinav 75 177-187 1969

The Institute of Clinical Bacteriology (Head S Winblad M D)
University of Lund General Hospital Malmo Sweden

STUDIES ON *YERSINIA ENTEROCOLITICA*

Bacteriophages Liberated from Chloroform Treated Cultures

By

BIRGITTA NILÉHN and CARL ERICSON

Received 21 vi 68

In 1965 *Mollaret & Nicolle* reported on bacteriophages obtained from culture filtrates or by UV induction of bacterial strains referred to as *Yersinia enterocolitica* (*van Loghem* 1944 1946 *Frederiksen* 1964) and with lytic activity against other strains within this group *Nicolle et al* (1967) assumed the occurrence of phage patterns useful for typing but no details were given on the host ranges of the isolated phages or the phage patterns of the investigated strains

Studies on phage liberation and phage sensitivity in organisms referred to this somewhat heterogeneous bacterial group may yield taxonomically useful information

The results of a recent investigation (*Niléhn & Sjostrom* 1967) suggested that *Yersinia enterocolitica* is fairly common in human infections This induced us to try to devise phage typing methods useful in epidemiological studies

This paper gives the preliminary results of investigations of bacteriophages liberated from *Yersinia enterocolitica* or related bacteria by treatment with chloroform by the method described by *Kjems* (1955) Ten phage preparations were used for the typing of 154 strains of different origins

MATERIAL

Bacterial Strains

Except for phage typing the trains u d ere selected am ng 37 strains obtained from different laboratori a *F t urel i X* (*Daniels* 1963 *Daniels & Goud u aar l* 1963) *Yersinia enteror liti a* (*Fre! ! n* 1964 or *Bacterium enterocolit ci i* (*Schleifstein & Coleman* 1943 rigin l i mb ers an l code numbers given in Tal l 1

The f llowing collecti n were u l f l erent purposes

Phage liberation C le N 1 3 *I* *n itu ty in chloroform essays* C le Nos 1 2 4 5 6 8 10 13 15 1 2 31 -37 *Determination of lyti s t f*

We are in lel ted t Ir f *F I* The V terinary Medical In tu to l h lm Dr W *Fr l r l en* R g al D p tr nt Aallorg of The St un Institut Copenhag n i l Pr f *H H M ll t* In titut Pasteur Pa f l indly placing strain at our di p l

The in estig t n wa supp rt l l grant from the *Medi al Fa ulty f th University f Lund S* len fr m *Alf ed Ö t rlunds St ftel e* and fr the *Su l h M l cal Resea h Council*

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Acta path microbiol scandinav [illegible] 177–187 1969

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meat extract broth culture (~ 2? C) of the PS The phage titre was determined after incubation at ~ 2? C and examination of the plates after 1 and 2 da s respectively The number of plaque forming units (pfu) per ml was calculated from counts on plates with plaque numbers nearest to 50/plate in each dilution series

Determination of Lytic Spectrum

Each phage preparation was tested against its PS and a standard set of test strains All test strains were kept freeze dried The phage preparations were diluted and distributed as described ab ve the test strain were pre incubated for 6 hrs at ~ ?? C before the plate were inoculated The plates were examined after one and 2 days incubation at ~ ?? C The results were graded as follows ++ (>50 plaques) + (20–50 plaques) ± (<?0 plaques) — no plaque formation) The activity of the phage preparation on the different strains was compared with that on the PS and the results were recorded according to the principles used by *Blair & Williams* (1961) for staphylococci a ++ reaction in the same (or higher) dilution as on the PS = 5 in a dilution 10–10 times more concentrated than on the PS = 4 in a dilution 10^3–10^4 times more concentrated than on the PS = 3 in a dilution 10^5–10^6 times more concentrated than on the PS = 2 very weak lysis = 1 no lysis = —

Phage Typing

Phage typing was carried out essentially according to the methods recommended by *Blair & Williams* (1961) for typing of staphylococci Incubation was however performed at room temperature (~ ?2 C) The strains to be tested were pre incubated in meat extract broth for 6 hours at ~ 2? C, before the plates were seeded The aim was to obtain a uniform thin confluent growth Phage suspensions giving near-confluent lysis of respective PS were used as a routine test dilution (RTD) If possible also suspensions with a higher phage titre i e 1000 × RTD or in the case of less concentrated phage stock preparations 100 × RTD or 10 × RTD were also used A phage typing apparatus according to *Lidwell* (1959) was used for the application of the phage suspensions to the plates The plates were examined after 1 and ? days with a loupe and a colony micros ope The results were recorded essentially according to the criteria set up by *Blair & Williams* (1961) for staphylococci

All tests were performed in at least duplicate

RESULTS

Bacteriophages liberated from chloroform treated cultures could be demonstrated by plaque formation on incubation of the test systems at ~ 22 C but not at 37 C In a few cases minute plaques occurred at 30 C but propagation was not attempted at this temperature The results of the 22 C series are given in Table 1 In several test strains different plaque types occurred In a few test systems i e phage deliver ing strain/set of test strains plaque production was not constant on repeated assay As shown in Table 1 29 out of 37 chloroform treated strains produced plaques on various test strains Ph... demonstrated in these test systems usually pr ved to be active against bacteri ecologically different from the phage producing strains However a few strains isolated from hare chinchilla and man p oduced phages active within the respective ecological groups

Phages were not isolated from 1 chinchilla strain (No 17) from 4 out of 6 indole positive strains (Nos 19 21 22 nd from 3 human strains (Nos 3. 36 37) the latter diverg[illegible] ...dole nega tive strains of human origin in that they [illegible] ...dly from d xylose and lactose (*Mähn* 1967 b *Mähn*

Concentrated phage preparations we

TABLE

Phage Production and Phage Sensitivity at 22 C in Various Groups of Yersinia entero
Thirty seven Strains Tested for Phage

Chloroform treated strains

Origin	Code No	Original No	Plaque 1	[illegible]	4	5	6
Dog	1	Becht 200	—	—	—	+	—
Pig	2	Dickinson ml O+	+	—	+	+	+
	3	Rampon	+	—	+	+	+
Hare	4	Daniels 1028	+	+	—	—	+
	5	Carpenter DRL 255	—	—	+	—	+
	6	Lucas 404	+	+	—	—	—
Chinchilla	7	*Daniels 905*	—	—	+	—	+
	8	Daniels 924	—	—	+	—	+
	9	Daniels 931	—	—	+	+	+
	10	Daniels 975	—	—	+	+	+
	11	Siegmann 268	—	—	+	+	+
	12	Becht 13/61	—	—	+	+	+
	13	Becht 18	—	—	+	+	+
	14	*Becht 51*	—	—	+	+	+
	15	Frederiksen I 71	—	—	+	+	+
	16	Frederiksen P 131	—	—	+	+	+
	17	Frederiksen P 76 (Knox)	—	—		—	—
	18	Frederiksen P 77	+	+			+
Guinea pig	19	Frederiksen P 413 (Borg Petersen)	—	—		—	—
Man	20	Albany 33114	—	—	+	+	+
	21	Albany 5819	—	—		—	—
	22	Frederiksen P 219 (Bojsen Møller)	—	—		—	—
	23	Niléhn MY 99	—	—		—	—
	24–31	Nilehn MY 1 2 3 13 60 62 65 75	—			+	+
	32	*Niléhn MY 58*	+		+	+	+
	33	Hässig 2533/24	+	—	+	+	+
	34	Wauters I	+	—	+	+	+
	35–37	Niléhn MY 39 66 79	—	—	—	—	—

+ = plaque formation V = variable results

phages from 10 bacterial strains isolated from dog pig hare chinchilla and man Phage preparations from 6 bacterial strains were each propagated on more than one propagating strain (2/1 2/13 4/6 4/24 13/4 13/6 18/6 18/24 32/1 32/13 32/25 34/1 34/13 34/25) Altogether 18 different phage preparations were obtained their approximative *concentrations (pfu/ml on PS) and lytic spectra are given in* Table 2 The propagation of phages from one bacterial strain on different propagating strains sometimes resulted in phage preparations with different lytic spectra On the other hand several phage preparations obtained from different strains behaved very similarly Thus phages liberated from one Belgian strain (No 34) and one Swedish strain (No 32) both of human origin showed essentially the same lytic activity as phages originating form a strain isolated from pig (No 2) The *same was true for phage preparations* isolated from one dog strain (No 1) one human indole positive strain (No 20) and one human indole negative strain (No 31) In several cases lytic activity of a propagated phage was seen also on the *phage producing bacterial*

1
colitica or *Related Organisms Tested with the Chloroform Technique of Kjems (1955)*
Liberation on 24 Strains within the Same Groups

formation on test strains													
8	10	13	15	17	18	19	20–23	24	25	26	28	31	35–37
−	−	−	+	−	−	−	−	−	−	−	−	−	−
−	+	+	+	−	\	−	−	+	+	+	−	−	−
−	+	+	+	−	\	−	−	+	+	+	−	−	−
−	−	+	−	−	\	−	−	+	+	+	−	+	−
−	+	+	+	−	\	−	−	−	+	+	−	+	−
−	−	+	−	−	\	−	−	+	+	+	−	+	−
−	−	−	+	−	−	−	−	−	−	−	−	−	−
−	−	+	+	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−		−	−	−	−		−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	+	+	+	+	+	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	+	+	+	−	−	−	−	−	−	−	−	−	−
−	−	−		−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	−	−
−	+	+	+	−	\	−	−	−	−	−	−	−	−
	+	+	+	−	−	−	−	+	\	−	−	−	−
−	+	+	+	−	−	−	−	+	\	−	−	−	−
−	+	+	+	−	−	−	−	+	+	+	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	−	−

− = no plaque formation Indole positive strains

strain In spite of attempts at repeated single plaque propagation more than one plaque type often occurred

Ten of the phage preparations were used for comparing the phage sensitivity of 154 strains of *Yersinia enterocolitica* or related organisms isolated from man dog pig hare chinchilla and guinea pig The results are given in Table 3 None of the phage preparations showed any lytic activity against the indole positive strains isolated from man or guinea pig The 3 biochemically diverging indole negative human strains 1 strain isolated from pig (No 3) and 2 from chinchilla (Nos 7 8) showed no lysis either Two chinchilla strains (Nos 17 18) initially negative varied in their phage sensitivity on repeated assay

With the aid of any of 3 phage preparations with grossly the same host ranges (34/1 32/1 2/1) 122 phage sensitive human strains of *Yersinia enterocolitica* were divided into a major group (I) of 108 Swedish strains and a minor one (II) of 11 Swedish strains 1 strain isolated by *Hassig* (1949) 1 by *Wyler* and 1 by *Wauters et al* (1965) A strain of canine origin behaved like the group 1 strains and a small

group of pig strains isolated by *Dickinson & Mocquot* (1961) showed the same phage pattern as the human group II. Two human strains (groups III and IV) differed in their phage sensitivity from all other human strains. Several differences in phage sensitivity were demonstrated between human strains and a small group of strains isolated from chinchilla (group V). Three hare strains included as test strains in lytic spectra (Table 2) also differed in phage sensitivity from human strains. The differences in phage patterns among the human strains were not found to be correlated with any epidemiological data.

Sixty strains of other species belonging to the family *Enterobacteriaceae* were not lysed by any of the phage preparations.

DISCUSSION

The findings in this study confirm the suitability of an incubation temperature below 30° C for phage studies of the *Yersinia enterocolitica* group (*Mollaret & Nicolle* 1965, *Nicolle et al* 1967).

In accordance with the results obtained with other techniques by the authors mentioned above, the present results indicate a rather high incidence of lysogens in this bacterial group. In the course of this study additional phages were isolated by UV induction or from culture filtrates from strains not liberating phages after treatment with chloroform. However, *Kjems* (1955) chloroform technique with the modifications described proved useful in a preliminary survey of phage sensitivity and phage production in these organisms.

Previous studies of this rather heterogeneous bacterial group have shown certain biochemical differences between strains of different origin, i.e. chinchilla, hare and man (*Daniels* 1963, *Daniels & Goudwaard* 1963, *Frederiksen* 1964, 1967, *Knapp & Thal* 1963, *Mollaret & Chevalier* 1964, *Smith & Thal* 1965, *Mollaret & Lucas* 1965, *Nilehn* 1967 a, b and others). Various strains seem to be more or less related with respect also to their antigen composition (*Frederiksen* 1964, 1967, *Winblad* 1967). The present results as regards phage sensitivity and phage production may underline the relation between certain strains isolated from man, dog and pig and observed also by *Nicolle et al* (1967), while differences in behaviour were found in the cases of bacteria isolated from chinchillas and hares. Three biochemically different human strains (*Nilehn* 1967b, *Nilehn et al* 1968) were found to differ from other human strains also in their sensitivity to chloroform liberated phages. The relation of various indole negative strains to indole positive strains initially described by *Schleifstein & Coleman* (1939) and later referred to as *Bacterium enterocoliticum* by the same authors (1943) has been debatable (*Smith & Thal* 1965, among others). In this connection it was interesting to note that phage preparations with grossly the same host ranges were obtained from one indole positive and one indole negative strain, both of human origin

This may suggest some rather close relation between these two bacterial strains

In contrast to the results obtained by *Mollaret & Nicolle* (1965) and by *Nicolle et al* (1967) several phage preparations showed lytic activity on the phage delivering strain after propagation Whether this may be related to the phage concentration environmental influences or to results of genotypic or phenotypic modifications of the phage during propagation is obscure

Differences in host range between phage preparations originating from the same bacterial strain but propagated on different strains may also be explained in several ways Multiple lysogeny of the phage delivering strain with the liberation of phages showing different host ranges or admixture of phages from the propagating strain may be the case Phage mutation host induced genotypic or phenotypic changes may also serve as possible explanations

Phage preparations obtained by this method may be impure even with the use of single plaque propagation Further purification and identification as well as more information on the stability of phage preparations suitable for phage typing are desirable As might be expected a few bacterial strains gave varying results when different colony clones were tested Analysis of the present results of phage sensitivity of human strains collected in this laboratory showed no correlation between phage patterns and epidemiological data concerning the strains It will however presumably be possible to obtain a fairly large number of phage preparations with different host ranges from which phages useful for epidemiological study may be selected

Phage typing of some strains of other species did not indicate any high degree of lytic activity outside the group The same was true for phages obtained by Nicolle et al (1967) with other methods An investigation of a larger number of strains of Yersinia pseudotuberculosis would however be of interest as *Nicolle et al* (1967) have reported some degree of inter species phage sensitivity between *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*

SUMMARY

Chloroform treatment of bacterial cultures incubated at 22 C proved suitable for surveying phage production in strains of *Yersinia enterocolitica*

Phage preparations were obtained active against different ecological entities within this not definitively classified bacterial group The host ranges of 18 phage preparations were investigated on a set of 12 test strains Ten phage preparations were used for the preliminary phage typing of 154 strains of different origins Six indole positive strains 3 biochemically different indole negative human strains 1 strain isolated from pig and 2 from chinchilla were not lysed by any of the phage preparations The 124 remaining human strains fall into

4 categories (108 14 1 and 1 strains respectively) One canine strain behaved like the majority of the human strains 5 pig strain showed the same phage patterns as the group of 14 human strains Chinchilla strains and hare strains differed in several respects from the human strains

One phage preparation isolated from a indole positive human strain showed the same host range as a phage preparation originating from an indole negative human strain

The phage preparations were not active against 60 strains of other species of the family *Enterobacteriaceae*, including a small number of *Yersinia pseudotuberculosis* strains and 1 strain of *Yersinia pestis*

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Acta path microbiol scandinav 75 183–190 1969

BRIEF REPORT

EFFECT OF RETICULO ENDOTHELIAL BLOCKADE ON EXPERIMENTAL MURINE INFECTIONS

By *Knut Nordstoga*

The ability of colloidal or particulate substances transiently to block the reticulo endothelial system and their influence on experimental endotoxin shock and the generalized Shwartzman reaction are well known Though recognized for many years (for references see *Jaffe* 1931 and *Thomas* 1957) the effect of reticulo-endothelial blockade on experimentally induced infections seems to have received little attention In the present paper a comparison is given of the influence of reticul endothelial blockade on experimental infections when organisms of different pathogenicity are used

Materials and Methods

The material comprises laboratory mice inoculated with *Escherichia coli* (experiment 1 64 mice) *Salmonella cholerae suis* (experiment 2 48 mice) and *Pseudomonas aeruginosa* (experiment 3 64 mice) The *Escherichia coli* strain was isolated from the faeces of a healthy mouse and the *Salmonella* strain was recovered from a septicaemic porcine case whereas the *Pseudomonas* strain was isolated from a mink that died during a serious outbreak of *Pseudomonas* infection This latter strain had a very high virulence for mice Equal doses to all animals in each experiment (0 1 ml 0 2 ml and 0 1 ml respectively of 24 hour old broth cultures) were administered subcutaneously In each experiment the animals were equally divided in the *thorotrast group* and the *control group* The thorotrast groups received 0 2 ml of thorotrast (a colloidal solution from Fellows Testagar Laboratories Detroit containing approximately 25 per cent thorium dioxide) intravenously immediately before inoculation Routinely the mice were opened and the spleen aseptically removed Cultures were made from the spleen of all animals that died

Results

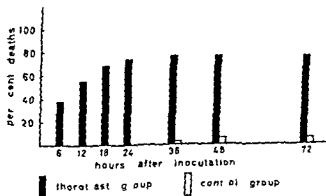


Fig 1
The difference in fatality rate in mice experimentally infected with *Escherichia coli* during the first 72 hours of the experiment

Received 13 xi 68 from the National Veterinary Institute Oslo

The first death in the thorotrast group occurred 3 hours after inoculation The spleen was congested and moderately enlarged in all animals that died, and *E coli* was cultivated from all splenic samples Surviving animals were killed 7 days after the beginning of the experiment (Fig 1)

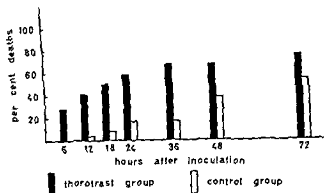


Fig 2

The fatality rate in experimental *Salmonella cholerae suis* infection during the first 72 hours after inoculation

In the thorotrast group the first mouse died 2 hours after inoculation All animals in this group died within 4 days while 3 mice in the control group were still alive after 9 days and were put to death The spleen was enlarged most evident in those that were killed and *S cholerae suis* was isolated nearly in pure culture from all cases (Fig 2)

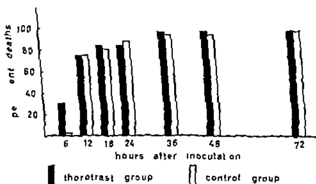


Fig 3

The mortality in mice experimentally infected with *Pseudomonas aeruginosa*

Using this highly virulent microbe the great majority of the animals in both groups died within 36 hours *Ps aeruginosa* was cultivated nearly in pure culture from all splenic specimens (Fig 3)

Discussion

The observations presented in Fig 1–3 clearly reveal that the effect of reticulo endothelial blockade is at its height in the early phase of experimentation This finding was expected since reticulo endothelial blockade is a temporary phenomenon which rapidly is followed by complete functional recovery When organisms of low pathogenicity (*E coli*) were used the distinct difference in fatality rates

Acta path microbiol scandinav 75 188–190 1969

BRIEF REPORT

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The material comprises laboratory mice inoculated with *Escherichia coli* (experiment 1, 64 mice), *Salmonella cholerae suis* (experiment 2, 48 mice) and *Pseudomonas aeruginosa* (experiment 3, 64 mice). The *Escherichia coli* strain was isolated from the faeces of a healthy mouse and the *Salmonella* strain was recovered from a septicaemic porcine case, whereas the *Pseudomonas* strain was isolated from a mink that died during a serious outbreak of *Pseudomonas* infection. This latter strain had a very high virulence for mice. Equal doses to all animals in each experiment (0.1 ml, 0.2 ml and 0.1 ml respectively of 24 hour old broth cultures) were administered subcutaneously. In each experiment the animals were equally divided in the *thorotrast group* and the *control group*. The thorotrast groups received 0.2 ml of thorotrast (a colloidal solution from Fellows Testagar Laboratories, Detroit, containing approximately 25 per cent thorium dioxide) intravenously immediately before inoculation. Routinely the mice were opened and the spleen aseptically removed. Cultures were made from the spleen of all animals that died.

Results

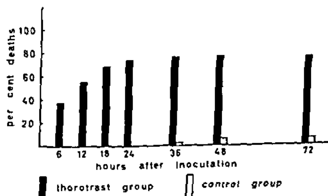


Fig. 1

The difference in fatality rate in mice experimentally infected with *Escherichia coli* during the first 72 hours of the experiment.

Received 19.xi.68 *from the National Veterinary Institute, Oslo*

Acta path microbiol scandinav 74 191-192 1969

BRIEF REPORT

A NEW CYSTIC FORM OF *TOXOPLASMA GONDII*

By K. Work and W. M. Hutchison

In 1965 *Hutchison* reported that he had been able to transmit *Toxoplasma gondii* in the faeces of experimentally infected cats and on the basis of his experiments he put forward the nematode transmission hypothesis (*Hutchison* 1967) which seemed to fit in well with the epidemiological facts Later in 1967 his results were confirmed by *Jacobs* and by *Dubey*

However in repeated experiments *Jacobs* (1967) reported discrepant results He was able to transmit *Toxoplasma* infections in faecal inocula from two cats without any nematode eggs being present About the same time *Hutchison* found that he was able to transmit the infection in the faeces of a cat in which the *Toxocara* eggs had not reached the infective stage These results led to a series of experiments in which it was clearly demonstrated that toxoplasmosis could be transmitted in the faeces of cats even in the absence of nematodes (*Hutchison et al* 1968) In these experiments however no idea as to the identity of the infective organism was obtained

After the completion of these experiments it was decided to repeat the work using different cats and a different *Toxoplasma* strain Thus the following additional experiment was performed Four cats all serologically positive with DT titres from 1 10-1 250 were each fed two chronically infected mice per day for seven days The mice had been inoculated about 10 weeks earlier with avirulent *Toxoplasma* (Statens Seruminstitut Strain 119) isolated from a pig Faeces from the cats were pooled and collected at six day intervals beginning on the first day of feeding No faeces were obtained prior to feeding After repeated filtrations and washings the sediment was subjected to flotation using saturated NaCl and 33 per cent $ZnSO_4$ After flotation the material was washed repeatedly and maintained in tap water for 3 weeks at room temperature In this way three inocula were obtained from three consecutive six day periods These inocula were microscopically examined at different stages of their preparation The first examination was performed immediately after flotation On this occasion it was noted that small cyst like structures were present in fairly large numbers in the inocula of the first and second period However none of these could be demonstrated in the inoculum of the third period The morphology of these cysts was apparently quite uniform They were ovoid measuring approximately 9 × 14 μ They were surrounded by a wall of uniform thickness and the interior was occupied by a slightly granular mass

Besides these cysts *Toxocara cati* eggs were present despite anthelmintic treatment of the cats prior to the experiment In order to exclude the possibility of nematode transmission the inocula were filtered through a 3 μ filter which separated the ova from these small cystic organisms No nematode ova could be demonstrated afterwards although the inocula were thoroughly examined on several occasions

About three weeks after collection of the faeces the material was inoculated into mice Examination under the microscope at this stage revealed a change in the interior of the cysts in which two separate organisms had developed At this stage they seemed to be morphologically identical measuring about 3 × 7 μ No definite structure except for some granules could be seen inside them

Each of the three inocula was given to a group of six mice 1 ml orally per mouse The mice were closely observed and in the course of 6-7 days those that had been inoculated with material from the first and second period were either dead or dying

Received 22 xi 68 from the *Department of Toxoplasmosis and Viral Diseases Statens Seruminstitut Copenhagen Denmark* and the *Department of Biology University of Strathclyde Glasgow Scotland*

Aided in part by grants to Dr J Chr Siim MD from *King Chr X Foundation US Publ Health Service Research Grant Division* (AI 03741) Bethesda Md *The Michaelsen Foundation* and to *Strathclyde University* by the MRC Wellcome Foundation and WHO

Acta path microbiol scandinav 75 193–200 1969

The Fibiger Laboratory Lyngby and the Dental Department
University Hospital Copenhagen and Department of Oral Pathology
Royal Dental College Copenhagen Denmark

STUDIES IN ORAL LEUKOPLAKIAS XVII

Lactate Dehydrogenase Isoenzyme Patterns in Oral Leukoplakia

By

E Langvad and B Roed Petersen

Received 8 viii 68

A high level of glycolytic metabolism is the main metabolic feature distinguishing most malignant tissue from the tissue of origin (*Warburg* 1930)

Direct assays of the glycolytic enzyme lactate dehydrogenase (LDH) in malignant and non malignant human tissue homogenates reveals that total LDH is increased in tumours (*Boxer* 1965 *Shonk et al* 1965) Recognition of the fact that LDH is present in multiple molecular variants (*Apella & Markert* 1961 *Cahn et al* 1962 *Dawson et al* 1964 *Kaplan & Ciotti* 1961 *Markert & Apella* 1961 *Markert & Moller* 1959 *Plagemann et al* 1960a *Plagemann et al* 1960b *Vesell & Bearn* 1957 *Vesell & Bearn* 1961 *Wieland & Pfleiderer* 1961) has led to a number of comparative studies of the LDH isoenzymatic pattern of malignant and non malignant tissues LDH isoenzymes are tetramers composed of four polypeptide chains which may be one of two polypeptides called subunit M and subunit H The five possible tetrameric combinations of these subunits (M_4 M_3H_1 M_2H_2 MH_3 H_4) exhibit different substrate affinities according to subunit composition Thus LDH_1 (H_4) is inhibited at pyruvate concentrations where the hybrid enzymes LDH (MH_3) LDH_3 (M_2H_2) LDH_4 (M_3H_1) and the antipode LDH (M_4) show increasing activity according to the relative content of M subunits

Since *Goldman et al* (1964) first reported an increased percentage of M subunits in malignant human tumours similar observations have been made by a number of other authors (*Gerhard et al* 1967 *Ishihara* 1968 *Langvad* 1968a *Langvad* 1968b *Latner et al* 1966 *Leese* 1965 *Nissen & Bohn* 1965 *Stanislawski Birencwajg & Loisillier* 1965 *Yasin & Bergel* 1965 *Langvad & Nodskov Pedersen* 1968) In recent studies

Sponsored by the *Danish Cancer Society*

This work was supported by the *Daell Foundation* and in part by a grant, DE 1358 to the Department of Oral Pathology from the *National Institute of Health* Bethesda Md. U S A

The authors are indebted to Professor E Husted M D Department of head and neck surgery University Hospital Copenhagen for supplying the material from oral carcinomas

whereas the mice that had been inoculated with material from the third period showed no clinical signs of illness. The survivors—three mice in the first group and five mice in the second group—were bled from the tail before dying and they all had developed a titre in the dye test of 1:10. The six mice in the third group as well as all the control mice were examined at the same time. They had all remained negative with a titre of less than 1:5.

These findings seemed to indicate a correlation between the presence of cysts in the inoculum, the clinical illness or death of the mice and the development of *Toxoplasma* antibodies.

In order to establish further evidence of a possible causal relationship between this cyst and *Toxoplasma* infection the following two preliminary experiments were conducted:

1) Preparation of a known suspension of cysts by counting and inoculation of serial dilutions into mice in order to establish a correlation between the number of cysts and the antibody formation in mice.
2) Microisolation of single cysts and inoculation of these into mice.

For these purposes a more concentrated suspension of cysts was prepared by repeated flotation and washing, and by means of a blood cell counting chamber a suspension containing 50 000 cysts per ml was prepared. Serial 10 fold dilutions were made and groups of six mice were inoculated with each dilution, each mouse was given 0.2 ml orally. Another six mice were kept as controls of each group. The six mice that received 10 000 cysts died or were dying from 8–11 days after inoculation. Blood was obtained from four: two had a dye test titre of 1:10 and two had 1:50. The corresponding control mice were negative with a titre of less than 1:5. In the group of mice that received 1 000 cysts one mouse died 10 days after inoculation and no serological examination was done. The brain of this and those of other mice that died were suspended in saline and each was inoculated into four fresh mice.

The surviving mice were bled and serologically examined 18 days after inoculation. The remaining five of the six mice that received 1 000 cysts were positive with titres of 1:250, and so were six mice that received 100 cysts each. A group of six mice was inoculated with 10 cysts and four of these were positive: two had a titre of 1:250 and two had 1:50. In two groups of mice that had been inoculated with one cyst and a dilution equivalent to 0.1 cyst respectively, all the mice were found to be negative. All control mice were tested at the same time and were negative.

Microisolation was performed with a Leitz micromanipulator. Four single cysts were isolated and inoculated intraperitoneally into four mice. Another four mice were used as controls. It had previously been shown (*Siim & Hutchison* 1966) that intraperitoneal inoculation of mice with faecal material could result in *Toxoplasma* infection. Eighteen days after inoculation the mice were bled from the tail and examined in the dye test. All four mice were positive with a titre of 1:250. The control mice were negative with a titre of less than 1:5. Microscopical examination of the brain of these mice was performed four weeks after inoculation. Numerous *Toxoplasma* cysts were demonstrated in the inoculated mice, whereas none could be seen in the control mice.

Summary

In the faeces of cats experimentally infected with *Toxoplasma* we have demonstrated a cystic organism which seems to be capable of producing *Toxoplasma* antibodies and *Toxoplasma* cysts in mice.

It seems most likely that a new cystic form of *Toxoplasma gondii* has been observed.

Acknowledgments

We wish to express our thanks to *J. Chr. Siim*, M.D. for many valuable discussions and suggestions during the performance of this work. We are also indebted to miss *E. Schwartz Møller*, mr. *F. Conradsen*, miss *E. Lauritzen*, mrs. *E. Matjeka* and mrs. *A. Schneefloth* for skilful technical assistance. Finally thanks are due to *H. J. Skovgaard Jensen*, D.V.M. for help and advice in the care of our animals.

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TABLE 1

Sex	Age	Biopsy site	Histopathological findings	LDHv/LDHs
Female	34	buccal mucosa	hyperorthokeratosis inflammation	39
	44	buccal mucosa	hyperparakeratosis atrophy inflammation	29
	44	alveolar ridge	hyperorthokeratosis atrophy inflammation	21
	45	alveolar ridge	hyperorthokeratosis inflammation	17
	46	buccal mucosa	hyperorthokeratosis hyperparakeratosis atrophy inflammation	2[illegible]
	47	alveolar ridge	hyperorthokeratosis inflammation	3[illegible]
	54	floor of mouth	hyperorthokeratosis atrophy	19
	59	buccal mucosa	hyperorthokeratosis hyperparakeratosis inflammation	14
	61	palate	hyperorthokeratosis atrophy inflammation	45
	6[illegible]	buccal mucosa	hyperorthokeratosis atrophy inflammation	12
	63	buccal mucosa	hyperorthokeratosis atrophy	33
	67	buccal mucosa	hyperorthokeratosis atrophy inflammation	20
	69	buccal mucosa	hyperorthokeratosis atrophy	63
	71	buccal mucosa	hyperorthokeratosis hyperplasia inflammation slight atypia	27
	76	commisure	hyperparakeratosis hyperplasia inflammation moderate atypia	34
	81	commisure	hyperparakeratosis hyperplasia	27
Male	47	alveolar ridge	hyperorthokeratosis	30
	61	commisure	hyperparakeratosis inflammation	45
	6[illegible]	buccal mucosa	hyperorthokeratosis hyperparakeratosis hyperplasia atrophy inflammation	35
	67	alveolar ridge	hyperparakeratosis hyperplasia inflammation	53
	67	commisure	hyperorthokeratosis hyperparakeratosis atrophy inflammation	33
	69	alveolar ridge	hyperorthokeratosis	53
	69	buccal mucosa	hyperorthokeratosis inflammation	15
	70	buccal mucosa	hyperorthokeratosis hyperplasia inflammation slight atypia	30
	74	commisure	hyperorthokeratosis atrophy inflammation	70
	77	alveolar ridge	hyperparakeratosis hyperplasia inflammation	47
	79	upper lip	hyperorthokeratosis hyperparakeratosis atrophy inflammation	20

Tabulated Histopathological and LDH Isoenzymatic Findings in 27 Patients with Oral Leucoplakias



Figs 1–2

Fig 1 Leukoplakia of right buccal mucosa in a male pipe smoker The surface of this type of lesion is clinically characteristic resembling pumice stone

Fig 2 Leukoplakia of left upper alveolar ridge and palate in denture bearing male The affection elevated above the surface of the surrounding mucosa is homogeneous and intensely white

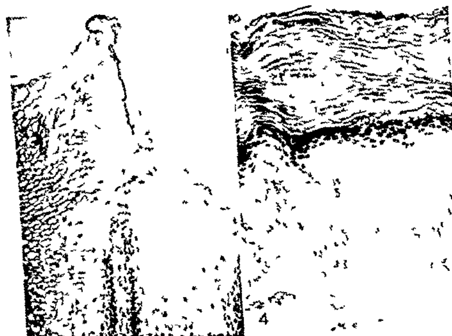
RESULTS

The basic data are presented in Table 1 from which it appears that the histopathologic picture was consistent with the clinical diagnosis (Figs 1–4) in all the biopsies from leukoplakias Among 14 normal control biopsies examined by light microscopy 4 specimens showed very slight parakeratosis but otherwise no pathological changes were seen

Age range of the patients with leukoplakia was 34 to 81 years and that of the normal control material 15 to 54 years The patients with oral carcinoma ranged in age from 66 to 81

The distribution of isonenzyme ratios for the leukoplakia and normal control material is presented in Fig 5 The mean ratio for leukoplakic tissue was 3 29 S D 1 50 while the mean value for control biopsies was 2 21 S D 0 85 It may be seen that the values were not normally distributed Hence it would be irrelevant to calculate a confidence interval Of the leukoplakic specimens 55 5 per cent had ratios of 3 0 or more while only 6 6 per cent of control biopsies showed ratios of this magnitude The results were subjected to the chi squared test which showed the difference to be significant ($x^2 = 13\ 97$ on 1 d f $P < 0\ 005$) The mean ratio of 6 oral carcinomas was 3 12 and did not differ significantly from the isoenzymes ratio of leukoplakic oral mucosa

No relation between isoenzyme ratio and age or sex of the patient could be detected Neither could any correlation be shown between the histological findings and the isoenzyme ratio However the highest



Figs 3–4

Fig 3 Photomicrograph of biopsy from patient shown in Fig 1 The surface consists of a one to two cell layer thick parakeratosis In the centre of the figure a cross-cut keratin ridge sends a thin strand of parakeratin into the epithelium This is as characteristic microscopically as is the pumice stone appearance clinically in this type of leucoplakia The outermost part of the prickle cell layer shows intracellular oedema No granular-cell layer is present The epithelium is hyperplastic cf Fig 4 (HE stain × 225)

Fig 4 Photomicrograph of biopsy from patient shown in Fig 2 The surface is made up of a pronounced hyperorthokeratotic layer A granular-cell layer is present The spinal-cell and basal-cell layers are of normal thickness In the connective tissue a slight inflammatory reaction can be seen (HE stain × 275)

LDH isoenzyme ratio 7 0 was found in the sole speckled leukoplakia included in this material

DISCUSSION

The isoenzymatic pattern obtained by electrophoretic separation of the supernatants of a tissue homogenate is the resultant of the individual patterns of all tissue elements present Inflammatory cells are regular contaminants of tissue biopsies from leukoplakias Lymphocytes and polymorphonuclear leucocytes have isoenzyme ratios which calculated on the basis of the results published by *Rabinowitz & Dietz* (1967) are 0 26 and 1 28 respectively Infiltration with polymorphonuclear leucocytes and lymphocytes would therefore tend to diminish the observed difference between the mean ratios of the leukoplakic and normal epithelium and can have no part in the differences observed



Figs 1–2

Fig 1 Leukoplakia of right buccal mucosa in a male pipe smoker The surface of this type of lesion is clinically characteristic *resembling pumice stone*
Fig 2 Leukoplakia of left upper alveolar ridge and palate in denture bearing male The affection elevated above the surface of the surrounding mucosa is homogeneous and intensely white

RESULTS

The basic data are presented in Table 1 from which it appears that the histopathologic picture was consistent with the clinical diagnosis (Figs 1–4) in all the biopsies from leukoplakias Among 14 normal control biopsies examined by light microscopy 4 specimens showed very slight parakeratosis but otherwise no pathological changes were seen

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The distribution of isoenzyme ratios for the leukoplakia and normal control material is presented in Fig 5 The mean ratio for leukoplakic tissue was 3.29 S.D. 1.50 while the mean value for control biopsies was 2.21 S.D. 0.85 It may be seen that the values were not normally distributed Hence it would be irrelevant to calculate a confidence interval Of the leukoplakic specimens 55.5 per cent had ratios of 3.0 or more while only 6.6 per cent of control biopsies showed ratios of this magnitude The results were subjected to the chi squared test which showed the difference to be significant ($\chi^2 = 13.97$ on 1 d.f. $P<0.001$) The mean ratio of 6 oral carcinomas was 3.12 and did not differ significantly from the isoenzymes ratio of leukoplakic oral mucosa

No relation between isoenzyme ratio and age or sex of the patient could be detected Neither could any correlation be shown between the histological findings and the isoenzyme ratio However the highest

nant tissue already have developed in leukoplakias These observations agree with the LDH isoenzymatic findings in other premalignant conditions such as intestinal metaplasia of the stomach (*Yasin & Bergel* 1965 *Leese* 1965) and chronic ulcerative colitis (*Langvad* 1968c) as well as with the findings of similar isoenzymatic changes in apparently non affected areas of tumour bearing organs (*Langvad* 1968 a b)

It is known that oral leukoplakia taken as an entity is a lesion of great chronicity Some lesions may regress completely others may remain unaltered while still others may progress into sudden malignization

The prognostic value of LDH isoenzyme determinations in oral leukoplakia cannot be evaluated from the present study Obviously a larger material and prolonged follow up studies are needed

SUMMARY

The lactate dehydrogenase (LDH) isoenzyme patterns of leukoplakic lesions normal oral mucosa and oral carcinomas have been studied in homogenates by disc electrophoretic separation

The cathodic shift of the isoenzymatic pattern characterizing neoplastic tissue also was found in leukoplakic tissue The findings are in agreement with the malignant potential of this affection and similar to the LDH isoenzymatic alterations seen in various other pre neoplastic conditions The present observations indicate that metabolic modifications facilitating a high rate of glycolysis occur at an early stage of advancing carcinogenesis where the development of morphological malignancy may still be one of several possible end points

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Acta path microbiol scandinav 75 201-219 1969

The Department of Pathology University of Uppsala Sweden

STUDIES ON THE EPIPHYSIAL GROWTH ZONE

III *Electronmicroscopic Studies on the Normal Epiphysial Growth Zone*

By

BENGT ENGFELDT

Received 7 vi 68

The epiphysial growth plate has been studied extensively under physiological and various pathological conditions using light microscopy and different histochemical procedures By this means we have in recent years greatly improved our knowledge of the complex changes which can occur in this area New techniques such an autoradiography and microchemical procedures on the tissue level have also been of great assistance in clarifying the nature of some of the structural elements (cf *Hjertquist* 1964) Further detailed information has been brought forth through electron microscopy (cf for example *Robinson & Cameron* 1956 *Godman & Porter* 1960 *Takuma* 1960 *Knese & Knoop* 1961 *Cameron* 1963 *Matukas et al* 1967)

In the following a survey of ultra tructural observations on the epiphysial growth zone of young normal rabbits will be given In this study some findings have been obtained which add to previous observations of other investigators and the results will also serve as a basis for further research on the structure of the epiphysial plate in animals subjected to treatment of various types

MATERIAL AND METHODS

Albino rabbits 3-4 weeks old and weighing 290-390 g were used The animals were anaesthetized and the proximal tibial epiphysis as well as the growth zone of the *ribs was dissected Thin slices taken in the longitudinal direction of the bone* were cut out under a dissection microscope Pieces of cartilage and neighbouring bone were immediately fixed The following fixation procedures were used

1) Fixation in 6 25 per cent glutaraldehyde in 0 075 M cacodylate buffer (pH 7 4) for 6 hours at +4 C, followed by *immersion in 0 1 M* cacodylate buffer containing 7 5 per cent sucrose pH 7 4 for 1 hour followed by *postfixation* in 1 per cent os*mium tetroxide* in 0 1 M phosphate buffer pH 7 4 for 2 hours followed by immersion in Tyrode s solution for 1 hour followed by immersion in 70 per cent 90 per cent and 95 per cent (v/v) aqueous ethanol absolute ethanol and propylene oxide for 1 hour in each solution (*Sabatini et al* 1963) The *pieces* of tissue were then embedded *in Epon*

2) Fixation in 6 25 per cent glutaraldehyde in 0 075 M cacodylate buffer con

taining 0.5 per cent cetylpyridinium chloride pH 7.4 for 6 hours. The subsequent steps were the same as those given above (cf. *Engfeldt & Hjertquist* 1968).

3) Fixation in 6.25 per cent glutaraldehyde in 0.075 M cacodylate buffer pH 7.4 for 6 hours followed by immersion in 0.1 M cacodylate buffer containing 7.5 per cent sucrose pH 7.4 for 1 hour followed by 0.1 M phosphate buffer pH 7.4 for half an hour. The pieces of tissue were then incubated in a similar phosphate buffer containing 0.05 mg of testicular hyaluronidase per ml for 18 hours followed by postfixation in osmium tetroxide. The subsequent steps were the same as those given under 1.

4) Fixation in osmium tetroxide in 0.1 M phosphate buffer pH 7.4 for 2 hours followed by immersion in Tyrode's solution for 1 hour and then 70 per cent ethanol overnight. Dehydration in a series of ethanols followed by propylene oxide for half an hour followed by embedding in Epon.

The blocks were trimmed and 1–2 micron thick sections were cut on an LKB ultratome fitted with glass knives. These sections mounted on glass slides were studied in a phase contrast microscope and some of them were stained with an aqueous solution of Azure A 10^{-3} molar pH 2.0 adjusted with hydrochloric acid. These sections were used as a guide when areas for ultrathin sectioning were selected.

The ultrathin sections taken for electron microscopy were examined unstained or after staining with uranyl acetate in a saturated aqueous solution followed by lead citrate (*Reynold* 1963). Microscopy was performed in a Siemens Elmiskop I operated at 60 kV.

RESULTS

When the different ways of preparation of the material for Epon embedding were used a comparison of the preservation of cellular and extracellular structures revealed that the best preservation of extracellular structures was obtained by fixation with glutaraldehyde to which 0.5 per cent cetylpyridinium chloride was added. In the report given here the observations refer to such material if not otherwise stated.

The ultrathin sections from the material prepared in different ways were studied both unstained and after staining with uranyl acetate and lead citrate. The contrast of the unstained sections was poor. Structures similar to those seen after staining were observed however.

In the epiphyseal growth zone we find towards the end of the long bone an area containing so called *resting cells*. This narrow zone comprises oval or spindle shaped cells located in a lacuna occupying most of its space. The cytoplasm contains quite a few mitochondria and there is a well developed endoplasmic reticulum often of a dilated type. The cell surface contains many fine cytoplasmic processes. The lacunar space outside the cell is narrow and contains small amounts of round granules and very fine fibrils. The matrix between the cell lacunae is built up mainly of a network of fine fibrils measuring 100–200 Å in diameter.

In the zone of proliferation the chondrocytes acquire a characteristic columnar arrangement (Figs. 1–4). The cells are still spindle shaped and the cytoplasm displays a prominent Golgi apparatus and many small vacuoles with rather low electron density are observed. The con-

The preparation contained 20000 T.R.U. per mg and was kindly provided by Dr. Hogberg, AB Leo, Hälsingborg.



Fig 1

The zone of young proliferating cartilage of epiphysial plate is shown in low magnification electron micrograph The ar angement of the cells in c nns is evident The cytoplasm contains a d l d endoplasmic reticulum and umerous vacuoles with l w el tron density × 4000

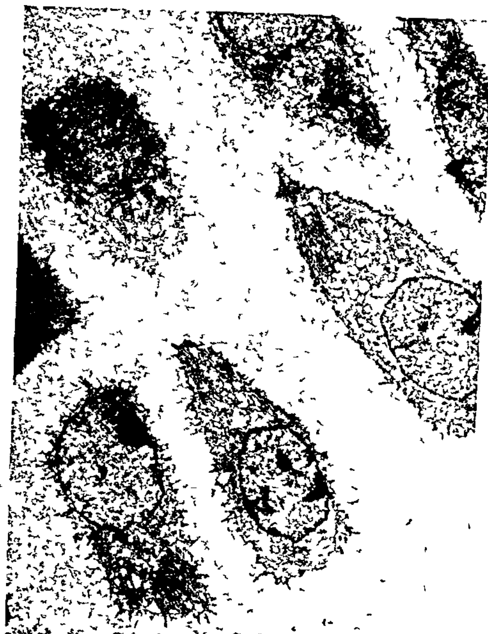


Fig 9

Area of the epiphysial plate high up in the hypertrophic zone The endoplasmic reticulum is prominent. Several vacuoles with low density are seen in the cyto plasm. Around the cells a narrow lacunar space is observed In this area round or oval electron dense granules are seen In the interlacunar space the matrix contains minute granules and very fine fibrils × 5000



Fig 3

A chondrocyte f om the me area hown in Fig ? to demonstrate the general architechture of the l cunar and inte lacuna spaces in this region × 12000

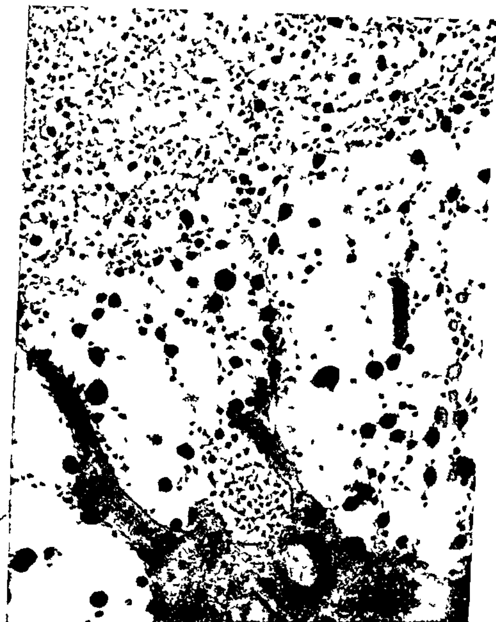


Fig 6

Electron micrograph of detail of the chondrocyte of Fig 5 In the middle of the picture a cytoplasmic vacuole is observed opening up into the matrix of the lacunar space The particles in this vacuole are of similar size as the granular material in the interlacunar areas and in some regions of the lacunar matrix The large dense bodies are almost exclusively located in the lacunar space outside the cell × 50000

tent of this vacuole resembles in its structure the granular material in certain extracellular areas. Some of these vacuoles are found close to the cell border and they are sometimes seen to open up into the extra-cellular space (Figs. 5 and 6).

In the lacunar area many fine cell projections are observed. Along the cell membrane oval or round electron dense particles measuring 600–1200 Å in diameter are seen. Such particles are also found in large amounts lying free in the lacunar spaces (Fig. 6). To these particles are often attached very fine fibrils approximately 15–30 Å in diameter (Fig. 6). In the lacunar area other fibrils measuring 50–200 Å in diameter are also observed. These fibrils reveal no characteristic collagen banding. Between the single cell lacuna and between the cell columns the matrix contains large amounts of fibrils of a collagenous nature (Fig. 5). No definite banding of these structures was observed. In this area the 600–1200 Å large round electron dense particles are largely lacking while many smaller (100–300 Å) oval or round less dense granules are seen. The shape of these structures is similar to the structure of contents in the above mentioned vacuoles of the cytoplasm.

In the just described area the cell lacuna enlarges accordingly as the metaphysis is approached gradually entering into the zone of *hypertrophic cells*. In this area the extracellular lacunar space is much larger but contains the same structural elements as those observed in the proliferation zone (Figs. 7–8). The cells are more rounded and the cytoplasm contains an endoplasmic reticulum which is more prominent than that in the previously described zone and furthermore large dilated cisterns are observed. Many long fine cytoplasmic projections are observed which sometimes reach the boundary of the lacuna.

In the *zone of calcification* concerning two to four cells above the invading metaphyseal capillaries the chondrocytes start to degenerate in places and the cell organelles are not so well defined (Fig. 9). Complete dissolution of the cytoplasmic content may occur leading to clear spaces within the cell. In the longitudinal interlacunar bars small areas with deposition of mineral salt are observed (Fig. 10). These areas are located in the centre of the bars away from the lacunar spaces. Passing downwards to the metaphysis the amount of calcium salts deposited increases rapidly and the bars are fully mineralized when the epiphysio-metaphysial border is reached (Fig. 11). In this area the mineralization is not found only in the centre of the bars but covers the whole interlacunar space. The transverse bars separating the individual lacunae have not been found to calcify. In the area where the mineralization starts the deposition occurs focally. The mineral salt *is deposited* [illegible] *in the form* of fine needles, the *short dimension* being 20–40 Å and the long 400–800 Å. When the mineralization became more widespread [illegible] the single crystallites could not be detected with the technique used.

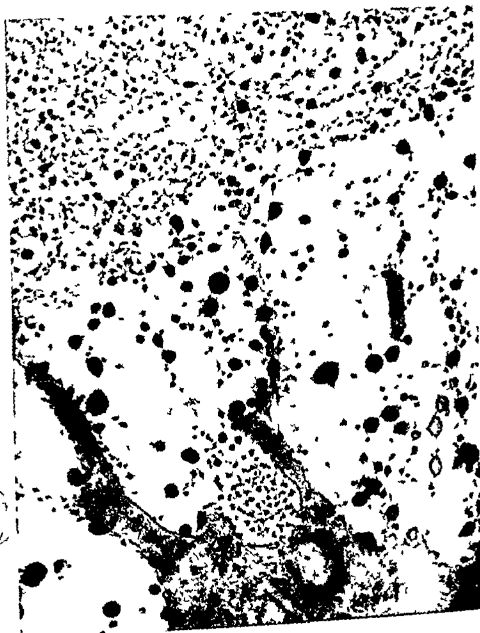


Fig 6

Electron micrograph of detail of the chondrocyte of Fig 5. In the middle of the picture a cytoplasmic vacuole is observed opening up into the matrix of the lacunar space. The particles in this vacuole are of similar size as the granular material in the interlacunar areas and in some regions of the lacunar matrix. The large dense bodies are almost exclusively located in the lacunar space outside the cell. × 50000

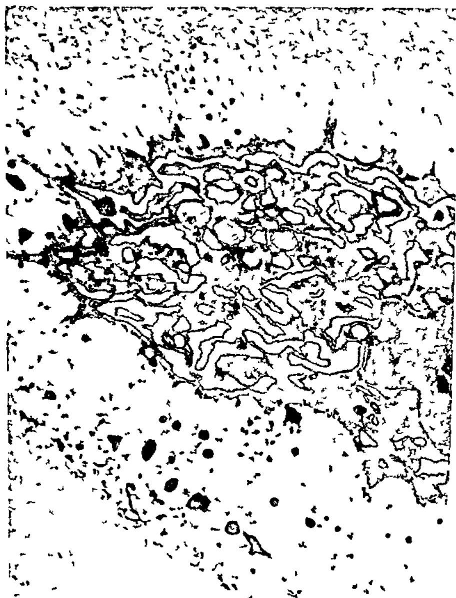


Fig 8

Fle t on mi r s l h d t l f a ch l [illegible] tes from the same area as shown in F g 7 In tl [illegible] me very l rge irre ular b dies lyin free or attached t th ll membr ne are l served S n e of these structures are also found in the bound [illegible] l t c n th l un [illegible] a c d interlacunar matrix but n t furthe n a l th l a s of th matrix × 13000

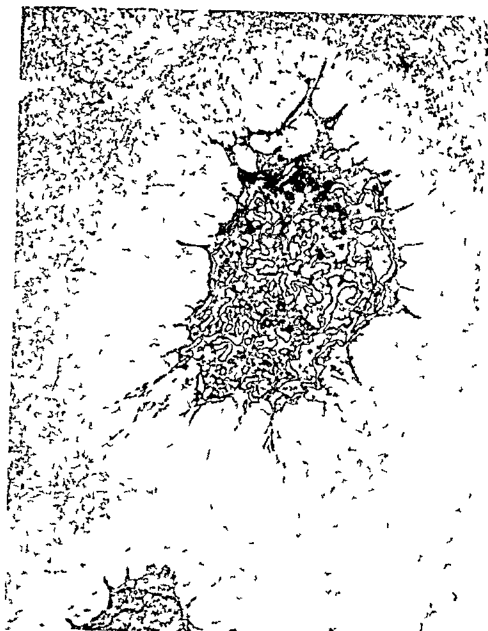


Fig 7

Electron micrograph showing cell lacuna with chondrocyte further down in the hypertrophic zone The lacuna is enlarged On the surface of the cell many fine cytoplasmic projections are observed These projections sometimes reach the border between the lacuna and the interlacunar matrix In the intralacunar space *outside* the cell abundant granules of different sizes are observed as well as very fine fibrils In the interlacunar matrix fibres of varying dimensions are seen × 5000

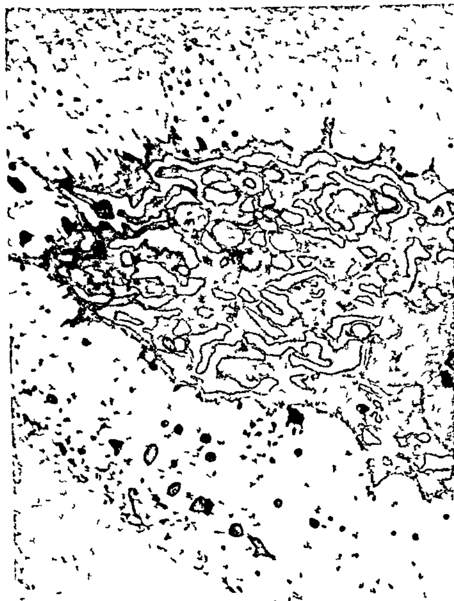


Fig. 8

Flc tr n micrograpl l [illegible] f [illegible] n tro ytes from the same area as shown in Fig 7 In the lacun [illegible] me [illegible] l rge irre ular bodies lying free or ttached t the cell m mt [illegible] S me of these structures are also f und in the b undar betw [illegible] and interlacunar matrix but n t further [illegible] the matrix × 13000



Fig 9

Electron micrograph of the epiphyseal plate from the area of hypertrophic cells in incipient disintergration Part of the cytoplasm of the chondrocyte is seen in the middle The lacunar space contains structures of the same type as described above × 6000



Fig 10

Electron micr[illegible] of lacuna and longitudinal bar from the upper part of th[illegible] zon[illegible] In the matrix small clusters of crystallites are seen [illegible] th[illegible] very beginning of mineralization. × 10000



Fig 11

Low magnification electron micrograph of the metaph[illegible] [illegible] the upper left a mineralized longitudinal bar covered by [illegible]te blasts The [illegible] contain a prominent endoplasmic reticulum and are rich in mit[illegible] [illegible] 4000 unstained

The findings described here are from a qualitative point of view similar with the different fixatives used However a reduction in the number of the large round electron dense granules of the lacunar matrix was observed both in the osmium fixed and in the hyaluroni dase treated material Hyaluronidase treatment did not however appreciably decrease the amount of material found in the cytoplasmic vacuoles of the proliferating and hypertrophic zones On staining of epon embedded sections with Azure A at pH 2 0 and observing them in the light microscope a definite decrease in the number of meta chromatic intralacunar granules was found in the material treated with hyaluronidase and also after osmium fixation This technique revealed no change in the metachromasia of the interlacunar space

DISCUSSION

The epiphysial growth zone is composed of cartilaginous cells with abundant matrix separating the individual cell The matrix contains large amounts of water collagen and glycosaminoglycans The water content of the epiphyseal plate is estimated to be approximately 80 per cent On a dry weight basis this area contains about 20 per cent col lagen and 5–6 per cent hexosamine (*Westerborn* 1961) The main part of the glycosaminoglycans is in the form of the 4 or 6 isomers of chondroitin sulphate probably as hybrids (cf *Hjertquist* 1964) Small amounts of hyaluronic acid are also present and probably also some keratan sulphate The glycosaminoglycans are present in the tissue as a protein glycosaminoglycan complex This complex is very large having an estimated molecular weight of several millions

In the present material the granules in the lacunar spaces observed with the phase contrast microscop stained metachromatically with Azure A at pH 2 0 This finding indicates that they contain sulphated glycosaminoglycans (cf *S irmat* 1963) These granules could also be identified in the material studied in the electron microscope They are represented by the large electron dense oval or round particles which appear at the cell surface and in the lacunar space outside the chondro cyte After treatment with hyaluronidase many of these structure are still present When this area is observed in the light microscope after treatment with hyaluronidas however a diminution or disappearance of the metachromatic reaction is noted It has been suggested that these dense structures represent deposited amorphous calcium phosphate However the density in unstained sections is far too low and soft X ray microscopy reveals no highly absorbing granules in the intralacunar space as would be the case if they were composed of calcium phosphate (cf *Hjertquist* 1964) Furthermore it has been shown in an electro- microscopic study of the epiphysial growth zone in rabbits treated with papain that the lacunar spaces are almost totally devoid of these large

granules (*Engfeldt* 1969) indicating that the proteolytic enzyme papain attacks these structures

These findings thus indicate that the granules seen represent for the most part a sulphated glycosaminoglycan protein complex Bio chemical analyses have demonstrated that the major part of the sul phated glycosaminoglycans is in the form of chondroitin sulphate and only traces of keratan sulphate are present Treatment with hyaluroni dase did not induce any marked qualitative changes of the granules as seen in the electron microscope But as the metachromatic reaction diminished or disappeared it is likely that the thin chains of chondroi tin sulphate linked to the protein molecules are split off whereby the granules loose their metachromatic reaction The granules are found exclusively outside the cell mainly in the lacunar space but in many instances attached to the cell membranes As no structures of this type were found to be lying free in the cytoplasm or in vacuoles this might indicate that at least the final aggregation of these structures takes place outside the cell

Obviously the possibility has to be kept in mind that the structure described is present *in vivo* in the tissue in some other form The treatment to which the tissue was subjected might have changed the molecules even if no loss of material took place hypothetically lead ing to some sort of upwinding of the molecular chains It is known that such an effect is brought about by for instance cetylpyridinium chlo ride It is worthy of observation however that the large dense bodies are found only inside the lacunar space while the metachromatic reaction indicates that sulphated glycosaminoglycans are present also n other parts of the matrix

The described dense granules thus seem to represent *one* structural form of the protein glycosaminoglycan complex present in epiphysial cartilage There are however several reasons to believe that this is not the only one It has thus been reported that after centrifugation of homogenized cartilage several fractions of the protein glycosamino glycan complex differing in sedimentation rates have been isolated (*Malawista & Schuberg* 1958) Furthermore it emerges from the pre sent study that the large dense granules seen in the lacunar spaces *in the proliferationzones* and *in* the hypertrophic zones do not occur to any appreciable extent outside the lacunae The interlacunar matrix is however also metachromatic at pH 2 0 In these areas fine fibrillar structures dominate the picture but are mixed with *rather dense but* much smaller granules 100–300 Å in diameter Also very fine fibrils seem to be attached to these structures This type *of structure* is also very similar to structures found in the intracytoplasmic vacuoles *and* they are also found here and there in *the lacunar space* Electron microscopic studies of hyaluronidase digested material revealed no marked alteration either in the structures *found in the vacuoles nor* in similar structures seen in the lacunae or in the interlacunar area

In the interlacunar spaces fine fibrils 100–200 Å in diameter were found. These fibrils probably represent tropocollagen and collagen. No banding was observed. It is however well known that if the banding is to be apparent it is necessary that the fibrils are composed of a certain number of molecular chains so that certain sites can be sufficiently electron dense to make them visible under the electron microscope. That the fine calibre is the reason for the absence of banding of the collagenous fibrils of epiphyseal cartilage also gets some support from findings in cartilage from other sources. In studies on hypertrophic cartilage from experimental fracture callus from rabbits it has thus been found that the fibres in the matrix have a considerably larger diameter and here the typical periodicity of collagen is also evident (*Engfeldt* 1968, to be published).

It has been reported in studies of young rats that round osmiophilic PAS reactive bodies occur in the calcifying cartilage at the periphery of the cells (*Bonucci* 1967). These bodies were supposed to represent the starting point of the calcifying process. Similar structures were found occasionally in the present material but were located in the matrix close to the lacunar space and not in the central area of the longitudinal bars where the first traces of calcification could be detected (cf. Fig. 8). The observations made in this study of the epiphysial plate from rabbits do not lend support to the above mentioned hypothesis.

The mineral salt is deposited as small needle shaped crystallites of a dimension of 20–40 Å in the short axis and up to 800 Å in the long axis. They are deposited in small clusters without any definite orientation of their long axes. In bone tissue the apatite crystallites are orientated with their long axes in the direction of the collagen fibres. The size of the apatite crystallites is about the same in these two locations and in both areas collagen is a major part of the matrix. However, in the calcifying cartilage of the epiphysial plate there is no evident orientation of the apatite crystallites.

The clusters of apatite crystallites increase rapidly in number and size towards the level of the invading metaphysial capillaries where the whole longitudinal bar is highly calcified. The reason why this pattern of calcification develops involves several, as yet unsolved problems. Possibly there might be a gradient in the concentration of calcium and phosphorus from the invading capillaries into the epiphysial plate. It has also been postulated that the disintegrating chondrocytes in this area are releasing substances which inhibit calcification (*Cameron* 1963). This inhibiting effect would then decrease with the disappearance of the cells. A depolymerization of glycosaminoglycans has also been supposed to result in calcification of the metaphyseal part of the epiphysial plate. This hypothesis has been put forward by *Glimcher* 1961 who also proposed that the substances of the matrix might bind calcium and that depolymerization could lead to local release of cal

granules (*Engfeldt* 1969) indicating that the proteolytic enzyme papain attacks these structures

These findings thus indicate that the granules seen represent for the most part a sulphated glycosaminoglycan protein complex Biochemical analyses have demonstrated that the major part of the sulphated glycosaminoglycans is in the form of chondroitin sulphate and only traces of keratan sulphate are present Treatment with hyaluronidase did not induce any marked qualitative changes of the granules as seen in the electron microscope But as the metachromatic reaction diminished or disappeared it is likely that the thin chains of chondroitin sulphate linked to the protein molecules are split off whereby the granules loose their metachromatic reaction The granules are found exclusively outside the cell mainly in the lacunar space but in many instances attached to the cell membranes As no structures of this type were found to be lying free in the cytoplasm or in vacuoles this might indicate that at least the final aggregation of these structures takes place outside the cell

Obviously the possibility has to be kept in mind that the structure described is present *in vivo* in the tissue in some other form The treatment to which the tissue was subjected might have changed the molecules even if no loss of material took place hypothetically leading to some sort of upwinding of the molecular chains It is known that such an effect is brought about by for instance cetylpyridinium chloride It is worthy of observation however that the large dense bodies are found only inside the lacunar space while the metachromatic reaction indicates that sulphated glycosaminoglycans are present also in other parts of the matrix

The described dense granules thus seem to represent *one structural form* of the protein glycosaminoglycan complex present in epiphysial cartilage There are however several reasons to believe that this is not the only one It has thus been reported that after centrifugation of homogenized cartilage several fractions of the protein glycosaminoglycan complex differing in sedimentation rates have been isolated (*Malawista & Schubert* 1958) Furthermore it emerges from the present study that the large dense granules seen in the lacunar spaces in the proliferationzones and in the hypertrophic zones do not occur to any appreciable extent outside the lacunae The interlacunar matrix is however also metachromatic at pH 2 0 In these areas fine fibrillar structures dominate the picture but are mixed with rather dense but much smaller granules 100–300 Å in diameter Also very fine fibrils seem to be attached to these structures This type of structure is also very similar to structures found in the intracytoplasmic vacuoles and they are also found here and there in the lacunar space Electron microscopic studies of hyaluronidase digested material revealed no marked alternation either in the structures found in the vacuoles nor in similar structures seen in the lacunae or in the interlacunar area

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ACKNOWLEDGEMENT

This work was supported by the Swedish Medical Research Council (Projects K 68 12x 540 04 and B 69-12x-540 05)

cium Microchemical analyses of the zone of calcifying cartilage have not however provided any evidence for depolymerization of glycosaminoglycans in this area (*Hjertquist* 1964) The mechanism by which calcification is brought about and limited to this specific area of the epiphysial growth zone is thus still unknown

SUMMARY

An account is given of the ultrastructure of the epiphysial growth zone in young rabbits The material was processed using different procedures to allow an estimation of differences in the preservation of the main structures of the matrix As postulated in an earlier theoretical consideration good preservation of tissue structures was obtained using fixation with *glutaraldehyde* to which 0 5 per cent cetylpyridinium chloride was added The ultrastructure of the different zones of the epiphyseal plate is described and compared *Evidence is presented that* the glycosaminoglycans protein complex is present in the epiphyseal plate in more than one structural manifestation The mechanism by which part of the epiphysial growth zone is calcified is discussed briefly

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Fig 4

Electron micrograph from epiph 1 l pl t f p p n treated rabl t l ing part of the hypertroph z ne with l ıı l eden t ll upying th l le lacı nar space In the interlacunar m t a n t (f f c fibrils is obs l × *12000*

period with polyornithine or with DEAE dextran would after subsequent incubation with heparin upon inoculation behave as untreated controls with regard to their ability to produce NJA leukaemia in C3H/A mice Tumour cell incubation with protamine sulphate had only slight inhibiting effect on tumour growth It was suggested that the tumour growth inhibiting mechanism might be functioning via an action on the cell membranes probably by changing the permeability of the membrane

The purpose of the present investigation has been to study the action of the high molecular polyanion dextran sulphate and to extend the study to include observations on other transplantable tumours in mice and rats

MATERIALS AND METHODS

Animals

C3H/A mice Maintained in the Cancer Research Institute Aarhus since 1950 by brother sister inbreeding

Balb/c mice Received from the Chester Beatty Research Institute London in February 1963 and maintained since then here in the institute

Wistar Rats Wistar/Mo/Ac were purchased from the breeding centre in Havdrup Denmark

Transplantable tumours

The NJA leukaemic tumour has been carried by subcutaneous transplantations by weekly transfers of approximately 10^6 tumour cells C3H/A mouse since it occurred as a spontaneous leukaemia in C3H/A mice in 1960 The tumour has been thoroughly described by *Olsen* (1964)

The JBI tumour used as an ascitic tumour in C3H/A mice Originally the tumour arose spontaneously in Ak/A mice (*Bichel* 1951) It has been maintained as ascitic tumour in the C3H/A mice since 1955

A plasmocytoma in Balb/c mice This tumour was kindly supplied t us by the Chester Beatty Research Institute London in 1963 It is characterized by the development of typical myeloma proteins in the serum of tumour bearing mice

Yoshida ascites tumours in Wistar rats were kindly supplied by the *Fibiger* Laboratory Copenhagen

Biological Testing

Solid tumours NJA/C3H/A and plasmacytoma Balb c were cli[illegible] with scissors and strained through gauze Cells were suspended in equal v lume f saline and N/10 phosphate pH 7 4 0 5 ml of suspensions containing 2×10^6/ml f tum ur cells were incubated with 0 5 ml containing the appr priate concentrati n f DEAE dextran (see Table 1) in the same saline phosphate buffer In order t ensure that each animal received the same amount of cells the incubati n to k pl c in disposable syringes All incubations with DEAE dextran were perf rmed at r m temperature for one hour Subsequent treatment of the cells with dextran sulphate was done b introducing into the same syringe the proper amount of buff r ntaining dextran sulphate and careful mixing f r 5 minutes at r m temperatur

Ascitic tumours JBI and Yoshida were treated in the ame was JBI ites fluid from tumour bearing C3H/A mice transplanted f 10 days in al an c ntaining about 100×10^6 cells ml and Yoshida cell w re washed twice with l ne and used in concentrations f 50×10^6 tumour cells ml

Evaluation of the effect of the tumour c ll incubation with DEAE dextran and dextran sulphate was ba ed on the b r ti n f the average ur i al time An increase in life span (ILS) b more than 40 per cent ver that f u treated c ntr l was taken as significant in rder to conf rm t the directi n gi en by *Skipper* & *Schmidt* (1962) Whenever it was p ssible t foll w the gr wth f tumour by weighings it was done This was nly b s ls rel vant in the ca e f the pr

TABLE 1

Effe t f in Vitro Incubation of Tun our (ells with DEAE Dextran and Subsequent Treatment with Dextran Sulphate on the Development of Different Experimental Tumours

Test Animal	Experimental Tumour	No of Animals	Treatment Incubation with Mg DEAE Dextran 1 hr	Mg Dextran Sulphate Subsequently 5 min	Survivors§	ILS of Dead
C3H/A mice	NJA leukaemia	80 (?)	0	0	0	
			1	0	all (30)	
			1	1 and 5†	0	
C3H/A mice	JBI ascites	90 (3)	0	0	0	
			1	0	29 of 30	+
			1	1	0	
Balb/c mice	Plasmocytoma	30 (1)	0	0	0	
			1	0	8 of 10	—
			1	3	0	
Wistar rats	Yoshida ascites	21 (1)	0	0	0	
			5	0	4 of 7	+
			5	10	1 of 7	—

Figures in brackets are number of series
† In one series 1 mg another 5 mg Dextran Sulphate
§ Survivors were observed for three times the average life pan of untreated control animals
Incre a ed life pan (ILS)
> 40 per cent +
less than 40 per cent —

period with polyornithine or with DEAE dextran would after subsequent incubation with heparin upon inoculation behave as untreated controls with regard to their ability to produce NJA leukaemia in C3H/A mice. Tumour cell incubation with protamine sulphate had only slight inhibiting effect on tumour growth. It was suggested that the tumour growth inhibiting mechanism might be functioning via an action on the cell membranes, probably by changing the permeability of the membrane.

The purpose of the present investigation has been to study the action of the high molecular polyanion dextran sulphate and to extend the study to include observations on other transplantable tumours in mice and rats.

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Yoshida ascites tumours in Wistar rats were kindly supplied by the Fibiger Laboratory, Copenhagen.

Biological Testing

Solid tumours NJA/C3H/A and plasmocytoma/Balb/c were clipped with scissors and strained through gauze. Cells were suspended in equal volume *of saline and* N/10 phosphate pH 7.4. 0.5 ml of suspensions containing 2×10^6 ml of tumour cells were incubated with 0.5 ml containing the appropriate concentration *of DEAE* dextran (see Table 1) in the same saline phosphate buffer. In order to secure *that* each animal received the same amount of cells, the incubation took place *in dis*posable syringes. All incubations with DEAE dextran were *performed at room tem*perature for one hour. Subsequent treatment of the cells with dextran sulphate was done by introducing into the same syringes the proper amount of *buffer containing* dextran sulphate and careful mixing for 5 minutes at room temperature.

Ascitic tumours JBI and Yoshida were treated in the same way as *JBI ascites fluid* from tumour bearing C3H/A mice transplanted 6–10 days *in advance containing* about 100×10^6 *cells/ml* and Yoshida *cells were washed twice* with saline and used in concentrations of 50×10^6 tumour cells/ml.

Evaluation of the effect of the tumour cell incubation *with DEAE-dextran* and dextran sulphate was based on the observation of the *average survival time*. An increase in life span (ILS) by more than 40 per cent *over that of untreated controls* was taken as significant in order to conform to the directions given by *Skipper & Schmidt* (1962). Whenever it was possible to *follow the growth* of tumour by weighings it was done. This was only *clearly relevant* in the case of the *pr*

does not enter ascites tumour cells Addition of DEAE dextran to NJA tumour cells influences the cellular oxygen consumption Heparin does not seem to counteract these effects of DEAE dextran *Larsen & Olsen* (1968)

If dextran sulphate do not enter the tumour cells the target for the tumour growth preventing action of DEAE dextran may not be directly on the DNA dependent RNA polymerase or other intercellular enzyme systems

The possibility exists that changes in cell surface charge and altered cell membrane functions are induced by interaction of the cell surface with polybases This has been suggested by *Katchalsky et al* (1959) and *Ryser* (1967) Polybases obviously have a great affinity for cell surfaces Primarily such an attachment of positively charged macro molecules would alter the cell surface charge This may be important in view of the possible relation between the negative surface charge of tumour cells and their decreased mutual adhesion lack of contact inhibition and increased invasiveness as suggested by *Ambrose et al* (1956) However the promoting action of polycations on diffusion of albumin through negatively charged gelatin membranes *Larsen* (1967) and the stimulating effect of polycations on the uptake of protein molecules into tumour cells demonstrated by *Ryser* (1965) may be observations which are of major importance The communication of the tumour cells with their environment will be changed if the uptake of proteins into the cells be stimulated (*Ryser* 1968) Although it is premature to suggest any connection between protein uptake and function of tumour cells it is tempting to presume that naturally occuring tumour inhibiting factors such as toxohormone or other factors demonstrated by *Olsen* (1963) *Holmberg* (1964) and *Szent Gyorgyi* (1965) may be more readily available for tumour cells which have interacted with polycations

SUMMARY

Tumour cells incubated *in vitro* for one hour at room temperature with DEAE dextran (diethylaminoethyl dextran) were unable to proliferate upon inoculation into the test animals or in a few cases were very slow to produce tumours This effect of DEAE dextran on the tumour cells was found to be reversible If the incubation with DEAE dextran were followed by 5 minutes of incubation with dextran sulphate the inhibitory effect would be reversed and the tumour cells treated in this way would behave as untreated tumour cells with regard to their ability to produce malignant growth in the animals The inhibitory effect of DEAE dextran on tumour growth and the reversability of this inhibition with dextran sulphate were demonstrated with four different experimental tumours NJA leukaemia in C3H/A mice JBI ascites tumours in C3H/A mice a plasmacytoma in Balb/c mice and with Yoshida ascites tumour in Wistar rats

incubation time with dextran sulphate is also sufficient for this reaction was not studied Figs 1 and 2 illustrate the actual course of the experiments with the NJA leukaemia tumour and the JBI ascites tumour In Fig 1 is depicted the differences in life span of mice in the control group inoculated with 10^6 NJA leukaemia cells and of mice in the group inoculated with the same number of NJA cells treated with dextran sulphate after one hour of DEAE dextran incubation All animals receiving NJA cells incubated for one hour with DEAE-dextran alone survive i e are found to be without demonstrable signs of tumours 45 days after transplantation 45 days are about 3 times the average life span of the controls Fig 2 shows the development of the JBI ascites tumours as registered by weighings of the mice Also here a clear inhibition of ascites production is obtained after incubation with DEAE dextran and subsequent treatment with dextran sulphate clearly reverses the action of DEAE dextran bringing the tumour cells back to their original activity The results from the experiments with the Balb/c plasmocytoma and the Yoshida ascites tumours in rats points in the same direction

DISCUSSION

In a discussion of the results, and on the basis of the present experiments attempt to localize the mode of action exerted by polycations in tumour cells the following propositions may be the most pertinent

It may be assumed that the RNA synthesis is influenced by basic macromolecules for instance by the mechanism suggested by *Allfrey et al* (1963), which involves inhibition of DNA dependent RNA polymerase with histones Accordingly competition with basic macromolecules and nucleic acid bound histones may take place

Inhibition of mitochondrial ATP ase systems due to interaction with basic macromolecules as described by *Schwartz* (1965)

Changes in cell surface properties adhesiveness invasiveness or membrane transport functions due to drastical alterations of the cell surface charge

It has been shown by *Ryser & Hancock* (1965) that there is a correlation between cellular uptake of basic polypeptides and proteins and the ability to stimulate cellular uptake of albumin DEAE dextran has a stimulating effect on the uptake of albumin into Sarcoma S-180 cells grown in monolayer cultures *Ryser* (1967) This may indicate that DEAE dextran is taken up by the cells and thus permit a reaction with intercellular constituents In order to understand the reversibility of the process as due to intercellular reactions also dextran sulphate would have to be taken up by the cells However there is considerable doubt whether such highmolecular anions do pass the cell membranes *Chapman Andresen* (1962) has shown that large anions in contrast to large cations do not stimulate pinocytosis in the amoeba *Chaos chaos* and according to studies by *Sherman* (1964) the polyanion heparin

does not enter ascites tumour cells Addition of DEAE dextran to NJA tumour cells influences the cellular oxygen consumption Heparin does not seem to counteract these effects of DEAE dextran *Larsen & Olsen* (1968)

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SUMMARY

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It is proposed that the inhibitory effect of DEAE dextran is due to alterations produced in cell membrane properties

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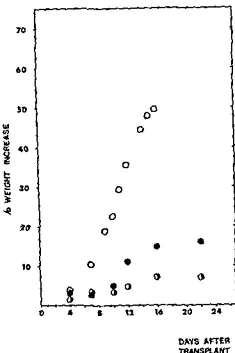


Fig 1

The effect of 1 mg of DEAE dextran on the weight increase in per cent of the pre transplantation weight Open circles Control mice inoculated i p with *10 × 10⁶ JBI tumour cells* Half closed circles Control mice given 1 mg of DEAE dextran Closed circles Mice inoculated i p with 10 × 10⁶ JBI tumour cells and subsequently given 1 mg of DEAE dextran after 6 hours

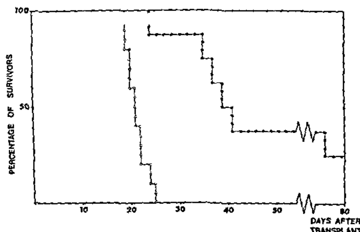


Fig 2

The effe t of 1 mg of DEAE dextran on the survival of mice inoculated i p with 10 × 10⁶ JBI tumour cells relating to Fig 1 Open circles Untreated mice (l d circles Mice given 1 mg of DEAE dextran i p 6 hours after the inocul t n

of the mice in the second experiment It is obvious that th weight gain is by far larger and faster in the controls than in the treated group

Fig 2 shows the survival data observed in this experiment It appears that the treated group survives twice as long as the untreated

the DEAE dextran The ascites tumour JBI was selected for the therapeutic trials of DEAE dextran

Competition between binding of the DEAE-dextran to cell surfaces and binding to proteins may be another factor of great importance The presence of the small amounts of ascites fluid protein does evidently not interfere with the *in vitro* inhibition whereas the inhibitory effect *in vivo* well might vanish completely as the probabilities of an adsorption of the DEAE dextran to tissue proteins are by far larger than otherwise required by the limited amount of material present during the incubation

The results obtained represent the preliminary approach to an evaluation of the therapeutic possibilities of DEAE-dextran as suggested by its tumour growth inhibiting effect observed in vitro

MATERIALS AND METHODS

C3H/A mice maintained at the Cancer Research Institute Aarhus by brother sister inbreeding since 1950

The JBI tumour Originally described by *J Bichel* (1951) It originated as a spontaneous tumour in the AK/A strain of mice at our Institute and has since been maintained in this strain Since 1955 it has also been transferred as solid and ascites tumour in the C3H strain

DEAE-dextran (diethyl amino ethyl dextran) is a soluble ion exchanger commercially available from Pharmacia in Uppsala This basic macromolecule has a molecular weight of about 2×10^6

In this series of experiments doses of 1 mg of DEAE were given in 1 ml of saline Only one dose of 1 mg was given Control experiments showed that this dose even if given for 5 days would not produce any signs of toxicity except for a slight and transient weight loss

EXPERIMENTAL PROCEDURES

The donor mice from which the tumour cells are obtained were transplanted 6–10 days earlier

The ascites fluid containing the tumour cells is diluted with saline to the appropriate concentration of cells per ml This tumour cell suspension is given intraperitoneally in doses of 0 2 ml per mouse

The growth of the tumour is observed by weighing every 2 5 days Removal of the ascites and reweighing showed that the weight gain of the mice is due to the production of the ascites and thus justifies this type of registration of the tumour growth although it is well known that the amount of ascites bears no strict relationship to the number of living tumour cells in the ascites fluid

RESULTS

In a pilot experiment it was shown that the injection of the DEAE dextran within 10 minutes after the tumour cell inoculation increased the survival time to twice the normal and that one out of 10 animals survived for 100 days without presenting any signs of tumour growth

It was decided to increase the interval between the two injections to 6 hours and in the following experiments this procedure was adhered to

Fig 1 demonstrates the influence of 1 mg of DEAE-dextran given 6 hours after inoculation of 10×10^6 tumours cells on the weight increase

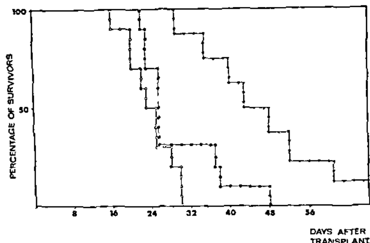


Fig 6

Survival data observed in the groups of mice demonstrated in Fig 5 Open circles Control mice inoculated with 10 × 10⁶ JBI tumour cells Closed circles Mice inoculated with *10 × 10⁶ JBI tumour cells* first incubated for 1 hour with 1 mg of DEAE dextran and subsequently for 5 minutes with ? mg of Dextran sulphate Open triangles Mice inoculated with 10 × 10⁶ JBI tumour cells previously incubated with 1 mg of DEAE dextran and subsequently 6 hours later injected i p with 2 mg of Dextran sulphate

In two experiments 10 × 10⁶ of DEAE-dextran treated tumour cells were given and Dextran sulphate injected 6 hours later The results from the first experiment are shown in Fig 5 *demonstrating the weight* increase in the various groups Fig 6 shows the survival data observed in the same experiment It appears that the mice given DEAE dextran *treated cells* exclusively did not develop tumours whereas such development was seen in 9 out of the 10 mice given Dextran sulphate Furthermore the figures demonstrate that DEAE dextran treated tumour cells subsequently incubated (in the syringe) with Dextran sulphate behave as untreated cells The tumours that developed in the Dextran sulphate treated mice were solid tumours *as opposed to the tumours in* the control animals This was not a constant finding and in the next experiment as shown in Fig 7 the dextran sulphate treated mice all developed an ascites tumour

In the first of these two experiments an additional group of 10 mice given DEAE treated cells was injected with Dextran sulphate after 7 days Two of these developed a solid tumour

In the second of these experiments a group of 10 mice given DEAE treated tumour cells received *injection of Dextran sulphate* after 24 hours Six of these mice came down with a solid tumour

In a third experiment the cell dose was reduced to 3 5 × 10⁶ As usual any tumours did not develop in the mice receiving the DEAE dextran treated cells Ninety per cent of the mice given a subsequent

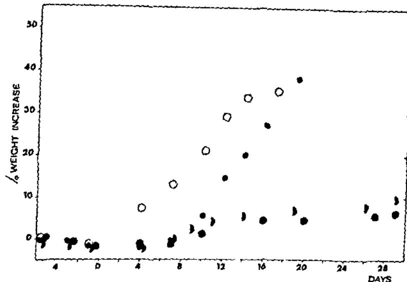


Fig 7

The weight increase in per cent of the pre-plantation weight demonstrating the Dextran sulphate induced reversion of the DEAF dextran inhibition *in vivo*. Open circles: Control mice inoculated with 10 × 10⁶ JBI tumour cells. Closed half circles: Mice inoculated with 10 × 10⁶ JBI tumour cells previously incubated for 1 hour with 1 mg of DEAF dextran. Closed circles small: Mice inoculated with 10 × 10⁶ JBI tumour cells previously incubated for 1 hour with 1 mg of DEAE dextran subsequently 6 hours later injected i p with 2 mg of Dextran sulphate. Closed circles large: Mice inoculated with 10 × 10⁶ JBI tumour cells previously inoculated with 1 mg of DEAF dextran subsequently injected i p 24 hours later with 2 mg of Dextran sulphate

i p injection of Dextran sulphate after 6 hours developed a tumour (ascites and/or solid). In those receiving the Dextran sulphate 24 hours after the inoculation of inhibited tumour cells tumours developed in 40 per cent of the cases

DISCUSSION

This series of preliminary experiments showed an unequivocal supressing effect of the polycation DEAE-dextran on the growth of an ascites tumour in the C3H/A strain of mice

It was possible by one single injection of 1 mg of DEAE dextran given six hours after the inoculation of the tumour cells to increase the survival time of the mice to twice that of the untreated controls and to completely prevent the growth of tumour in 20 per cent of the animals

Richardson (1959) found a similar effect of synthetic polylysine on the growth of the Ehrlich ascites tumour. According to *Shah & Reilly* (1967) incubation of tumour cells with polylysine was found to restrain the growth of a mammary carcinoma in mice

We have previously shown that incubation with DEAE dextran would inhibit the growth of various tumours. The present paper however is the first report of the inhibitory effect of this polycation *in vivo*

As to the mechanism of action of this polycation it is probably of major significance that the inhibitory effect can be reversed by subsequent contact with the polyanion Dextran sulphate. We have previously shown that this was possible *in vitro* and in the present work such effect could also be demonstrated *in vivo*.

Whereas it is conceivable that the DEAE-dextran may penetrate into the cancer cells and exert its effect on some intracellular component it is unlikely that the polyanion Dextran sulphate will enter the cell (*Sherman* 1964). Therefore, as Dextran sulphate will obliviate the effect of DEAE dextran it is most probable that the action of DEAE dextran is in some way connected with changes in the cellular membrane. According to *Ambrose* (1956) the tumour cells are heavily negatively charged on the surface. It is suggested that this property is of some significance for the specific malignant behaviour of these cells for instance their invasiveness and lack of contact inhibition.

It may also be that the cancer cells are in some way deprived of some informational signal from the surrounding normal tissue. In this connection the tissue chalones as defined by *Bullough* might be mentioned (1965) or the Dipersin previously described in our institute (*Kai Olsen* 1963). It is conceivable that the neutralization of part of the negative charge on the cell surface may re-establish the cell to cell contact allowing the cell to receive a signal to stop dividing. In this connection it is noteworthy that *Richardson* (1959) observed a decreased mitotic index of the tumour cells in the ascites fluid from mice treated with polylysine. The effect is probably rather local confined to the peritoneum. We measured the 48 hours Fe^{59} incorporation into red cells of mice after 1 mg of DEAE dextran and no decrease was observed indicating that the mitotic rate of the red cell precursors in the bone marrow is not affected. According to *Bullough* (1965) the tissue chalones are decreased in damaged tissue. This may be an explanation of the tendency of solid tumours to develop in the scar at the site of injection as seen in the case where the Dextran inhibited cells were reversed and also in the therapeutic experiments.

We consider it of major significance that the inhibited cells could be reversed after 24 hours or perhaps after intervals covering up to one week. It is an intriguing question what these harnessed tumour cells are doing in this interval. They are evidently not capable of establishing a malignant tumour but obviously they are not dead.

The action of the dextran sulphate is somewhat obscure. The complex of the two differently charged macromolecules may be split off from the cell surface restituting the conditions ad integrum or totally different mechanisms may be involved.

SUMMARY

According to the results here presented the polycation DEAE-dextran was found to have an inhibitory effect on the growth of an ascites tumour in C3H mice The DEAE dextran was given in a single dose of 1 mg 6 hours after the inoculation The survival time was doubled and 20 per cent of the mice did not develop a tumour

In vitro inhibited tumour cells would not establish a tumour after inoculation It is demonstrated that the inoculated inhibited tumour cells can be revived by a subsequent single injection of 2 mg of Dextran sulphate given up to 24 hours later

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Acta path microbiol scandinav 75 247–256 1969

The Institute of Pathology Bispebjerg Hospital Copenhagen Denmark
Head Charles Johansen M D

ADENOMAS OF THE SMALL INTESTINE

A Report of Four Cases with Special Reference to their Relation to Carcinomas

By

AAGE JOHANSEN and ELLA LARSEN

Received 29 vii 68

The statement that intestinal adenomas may be the precursor of carcinomas has been based on several arguments of which the most striking undoubtedly is the histological similarity presented by the tumours

In adenomas papillomatous polypoid and nonpolypoid the glandular epithelium shows a regular arrangement approaching that of the normal epithelium The different evaluations of the criteria for the diagnosis of malignancy probably is responsible for the different frequencies with which malignant changes are reported Such criteria are nuclear pleomorphism and atypism the arrangement of the nuclei in more than one layer the disappearance of the goblet cells the occurrence of an increased number of mitoses and more convincing the presence of atypical mitoses and the invasion of the muscularis mucosae It is surprising how seldom convincing malignancy is found in the common polyps

The above mentioned is far more valid for the colon but the problem is also relevant to the small intestine According to several authors (*Raiford* 1932 *Geschickter* 1935 *River* 1956) adenomas are the most common tumours in this part of the bowel too However the opportunity to study them and their possible malignant transformation is to the individual pathologists very limited The following report will comment on different sorts of polyps in the small intestine and particularly their relation to carcinomas will be discussed

CASE REPORTS

Case 1

43 year old woman previously well For the last two years before the actual admission she had suffered from uncharacteristic dyspepsia with epigastric pains ructus and pyrosis At admission the sedimentation rate was normal the haemoglobin was 1[illegible]5 g per cent In one of three examinations the benzidine reaction showed a slight positive reaction The X ray examination of the stomach revealed a duodenal polyp and a slight gastro enteritis

On March 2[illegible]th 1965 the patient was operated. In the proximal part of the duodenum a large polyp was found There was no infiltration of the duodenal wall and



Fig 1

Case 1 A A representative part of the centre of the adenoma (× 120) Inset The duodenal adenoma (× 2) B *The adenomatous pattern in the periphery (× 120)* C Invasion of the muscularis mucosae (× 180) (H&E)

the polyp was removed with half a centimeter of the mucosa round the stalk The postoperative run was uncomplicated

Pathological Examination

Macroscopic examination revealed a polypoid sessile tumour about 2 cm in diameter It was surrounded by a narrow edge of normal intestinal tissue Microscopically the tumour was composed of tubules which in the periphery (Fig 1 B) revealed a typical adenomatous pattern with regular cells with preserved polarity and only showing nuclear hyperchromatism and reduplication The goblet cells had disappeared Most of the tubules especially in the central part were closely packed and were lined by cells disclosing a much more pronounced pleomorphism (Fig 1 B) In many places they had lost their polarity and often their nuclei were very large and showed all signs of atypism A great number of atypical mitoses were seen By tripsections it was revealed that the atypical tubules had invaded and in a small area pervaded the muscularis mucosae (Fig 1 C) A scanty stroma was found it was heavily infiltrated with leucocytes and plasma cells No Brunner glands could be identified among the atypical tubules and nearly *all these glands* had disappeared in the submucosa too although they were found to be normal in sections from the edge of the specimen No lymph nodes were received for examination

Case 2

This case was the most interesting and will be given in more details The patient was a man aged 73 Due to senile dementia he had spent the last ten years of his life in a geriatric department With reference to the actual disease his past history was negative especially he had never suffered from billiary attacks On December 24th 1966 fresh blood on the stools was observed and a few days later the patient developed diarrhoea and vomitings X ray examinations gave suspicion of a carcinoma of the colon and he was transferred to this hospital

Physical examination disclosed a seriously ill patient The haemoglobin was 8 6 g per cent The sedimentation rate was 30 mm/hour The abdomen was distended and tender but nothing abnormal was palpated Repeated X ray examinations revealed ileus of the small intestine The patient was operated upon on January 9th 1967 The abdominal cavity contained about 2 litres of ascites fluid Heavy adhesions were found around the gallbladder which could not be palpated About 30 cm of the terminal ileum was saccularly distended and contained 5 large gallstones In the mesentery a lymphomalike tumour 6 cm in diameter was seen A frozen section taken from this tumour revealed granulation tissue without any signs of malignancy

A resection of the distended part of the intestine was performed The operation was uncomplicated but a few hours postoperatively the patient died

A complete postmortem examination was performed Apart from several small yellow plaques on the serosal surface of the rest of the ileum the whole intestinal tract was normal No perforation could be disclosed anywhere The above mentioned tumour in the iliac mesentery was soft yellow with some bleeding on section No enlarged lymph nodes were seen The gallbladder was small shrunken and surrounded by heavy adhesions No fistula was seen In the common bile duct which was moderately distended a dozen small dark gallstones each measuring 5 mm in diameter were dis losed A typical enlarged hyperplastic spleen was found. The pancreas was normal In both kidneys some small simple cysts were disclosed A heavy pulmonary oedema in combination with bronchitis lung emphysema and se ere coronary sclerosis were considered the cause of death

Pathological Examination

Macroscopically The surgical specimen showed a 28 cm long small intestinal loop which in the distal half was distended to 15 cm in circumference terminating in a slightly stenosed resection border In this half the mucosa was injected slightly granular and ulcerated and seven irregular but well defined pedunculated polyps were disclosed (Fig 2 C) They measured about one cm were elevated dark and fragile A larger and more sessile one of the same type was seen in the middle of the specimen Among these polyps especially near the stenosed resection border eight wartlike areas each measuring from 4 to 7 mm in diameter and elevated only 1 or 2 mm were found They were light in colour and had a fine granular surface On section no stalk could be seen The other half of the specimen was of normal dimensions The mucosa was normal with the exception of four polyps of the same appearance as the first mentioned above The whole intestinal wall was thickened measuring 8 mm near the resection border The peri intestinal fat was abundant On the serosal surface small yellow plaques a few mm in diameter were seen No lymph nodes were found in the mesentery

Microscopic examination revealed that the twelve irregular polyps including the big one in the middle of the specimen all were composed of a granulation tissue rich in leucocytes and with a great number of



Fig 2

Case 2 A Sessile adenoma of the ileum (× 1?0) B High p[illegible]wer [illegible] f the tubules. Tripolar mitoses are seen in three crypts C The highly vascul[illegible]r[illegible]ed granulation tissue of the inflammatory polyps (× 4?0) In[illegible]et One [illegible]f th[illegible] inflammatory polyps (× 2) (H&E)

capillaries (Fig 2 C) In some areas the tissue had a certain resemblance to a haemangioma All the polyps were ulcerated and devoid of surface epithelium but reminiscences of normal crypts were seen in some By further examination it was disclosed that this highly vascularized granulation tissue was found in many places in the intestinal wall including the serosal surface corresponding to the yellow plaques The tumourlike process in the mesentery had the same microscopical appearance

Sections from the wartlike polyps however disclosed a different picture (Fig 2 A) Very abruptly the normal intestinal epithelium changed to a neoplastic type The goblet cells disappeared completely and the nuclei assumed a highly atypical appearance They were hyperchromatic enlarged and often arranged in two or more layers In three out of these eight polyps nuclei with atypical mitoses were seen (Fig 2 B) In the other five an increased number of normal mitoses was found The adenomas were only slightly elevated above the level of the surrounding mucosa Signs of invasion of the muscularis mucosae were not found in any of the polyps

Case 3

69 year old woman previously well For five months before this admission she had been suffering from fatigue anaemia and loss of weight The laboratory findings showed positive reactions for blood in the stools the sedimentation rate was 21 mm/hour The haemoglobin 11 8 g per cent An augmented histamine test showed 18 meq/hour X ray examinations of the stomach and duodenum revealed a large defect in the duodenum A Billroth II resection was performed A papillomatous tumour was found situated in the first part of duodenum 1–2 cm distal to the pylorus The patient was discharged in good health

Pathological Examination

The specimen consisted of the pyloric part of the stomach it was without pathological changes Distal to the pylorus a polypoid tumour measuring 5 by 4 by 2½ cm was seen It was rather sessile and sharply demarcated from the surrounding duodenal mucosa

Microscopic examination (Fig 3) revealed a polypoid adenoma composed of a scanty stroma of connective tissue infiltrated with inflammatory cells The tubules were lined with columnar cells the hyperchromatic nuclei of which were arranged in two or more layers A complete loss of goblet cells was revealed Several normal mitoses were disclosed but no atypical ones were found Paneth cells were seen only in the tubules near to the muscularis mucosae No signs of invasion were found The Brunner glands and the surrounding mucosa were quite normal

Case 4

44 year old woman With reference to the actual case her past history was negative until 1967 when she was admitted to the surgical department for bleeding ulcer On October 3rd 1967 she was operated upon A 50 cm long invagination of the small



Figs 3 4

Fig 3 Case 3 Part of the adenomatous polyp (× 60) (H&E)
(Compare with Fig 4)

Fig 4 Case 4 Part of hamartogenous polyp (× 60) (H&E)

intestine was found situated 85 cm distal to the Treitz ligament In relation to the invagination a polypoid tumour was found The invagination was reduced and the tumour removed

Pathological Examination

Macroscopically a polypoid slightly pedunculated tumour about 4 cm in diameter was seen It was of a greyish red colour and was surrounded by a narrow edge of normal intestinal tissue Histological examination (Fig 4) disclosed that the stroma in the polyp was abundant and besides connective tissue contained branching bands of smooth muscle thickest in the centre becoming thinner towards the periphery The tubules and glands were lined by a normal intestinal epithelium No hyperchromatism could be disclosed and the number of goblet cells was not reduced Furthermore Paneth cells were seen everywhere also in periphery The number of mitoses in the tumour tissue was not greater than that in the surrounding jejunal mucosa

DISCUSSION

Papillomas and polyps of the intestinal tract are usually considered histologically to be adenomas According to *Machella* (1964) they are found in the small intestine with equal frequency in the duodenum and the ileum and a little more seldom in the jejunum As a rule they are small only a few mm in diameter and they usually are asymptomatic and are discovered incidentally at autopsy Larger ones may cause symptoms as illustrated by case 1 3 and 4 Their clinical importance is limited except for their possible malignant transformation

The three first reported cases illustrate varying degrees of malignancy in adenomas of the small intestine The histological changes in the first case are the most convincing ones since invasion of the muscularis mucosae has taken place The possibility that the tumour is a primary adenocarcinoma of papillary type cannot be absolutely rejected but is not likely since the microscopic examination disclosed areas with typical adenomatous patterns Furthermore by macroscopic inspection the surgeon as well as the pathologist had interpreted the tumour as a common sessile polyp The nearly complete disappearance of the Brunner glands in the submucosa is remarkable but as pointed out by *Robertson* (1941) and *Landboe Christensen* (1944) the density of the glandular distribution in the duodenum is highly variable The Brunner glands are not included in the tumour tissue which according to *Machella* (1964) is the case in fifty per cent of duodenal adenomas

Three of the sessile adenomas in case 2 can also be accepted as being in malignant transformation on account of the occurrence of highly atypical mitoses indicating a growth beyond control In the other five adenomas in this case the changes are fundamentally the same as those in case 3 These changes are the same as the ones usually found in the

far more common polyps of the colon namely nuclear hyperchromatism reduplication of the nuclei increased number of normal mitoses and a more or less complete disappearance of the goblet cells Such changes are neoplastic in nature but to what degree they are an expression of early malignancy whether in the colon or the small intestine is a question that remains to be solved

The literature concerning malignant transformation of benign adenomas of the small intestine is restricted According to *Machella* (1964) it has been estimated that about seven per cent of adenomatous polyps become malignant In comparison with this according to the same author about twenty per cent of leiomyomas develop malignant changes *Scianta & Volta* (1956) and *Sawyer et al* (1963) each reported on a duedenal adenoma with early malignant changes *Wieners* (1966) stressed that malignant changes are more likely to occur in great sessile polyps In this report all the adenomas with convincing malignant changes are of the extremely sessile type

A different histological appearance is found in case 4 The epithelium covering the tubules is of normal type and goblet cells and even Paneth cells are seen everywhere in the tumour So far it might be looked upon as an adenoma without the slightest malignant transformation However another striking thing is the occurrence of smooth muscle fibres extending to the periphery of the branching stroma This phenomenon endows the tumour with an appearance well consistent with the polyps of Peutz Jeghers syndrome but no mucocutaneous pigmentations were found in the patient According to *Bartholomew et al* (1957) such solitary polyps are sometimes found in the small intestine especially *in the jejunum Just as the polyps in Peutz Jeghers syndrome* these latter polyps may be looked upon as hamartomas a view supported by *Morson* (1962) Potential malignancy in these polyps is to be regarded as being of the same degree as in polyps of this syndrome The presence of glands among bundles of smooth muscle have given rise to a false appearance of invasion and although convictive malignant polyps in Peutz Jeghers syndrome have been described (*Machella* 1964) the precancerous significance of such polyps is still discussed Many authors (*Cormany* 1957 *Rintala* 1959) believe that such changes are exceedingly rare

The nonadenomatous irregular polyps mentioned in case 2 have an appearance very similar to that of a series of inflammatory polyps reported by *Klepinger & Pontius* (1964) These authors consider such polyps as simple exuberances of inflammatory granulation tissue rising above the mucosal surface and designated them pyogenic granulomas Since the greater part of the polyps in their series was situated in the rectum they believe that stercoral traumas may have some aetiological importance Similar cases have been reported by *Roth & Helwig* (1963) In our case it seems probable that the gallstones may have caused the inflammation and the polyps The presence of the granula

tion tissue of the serosal surface of the ileum and especially in the iliac mesentery is difficult to explain It seems as if the tissue has extended along the lymphatics in a tumour like fashion In some areas the tissue resembles a haemangioma but still there is no doubt about its inflammatory character No signs of perforation could be disclosed in the surgical specimen and nothing in the case history could be interpreted in this way

Although according to *Tumen* (1964) the most common site of gallstone ileus is the lower ileum also in cases where the bowel is normal it seems likely that the adenomatous polyps have been the direct cause of the obstruction They have not narrowed the lumen to any appreciable degree but they may have interfered with the peristaltic movement As reported they were all localized near to the stenosed resection border The gallstones had certainly not caused the adenomas but they may have accelerated the malignant changes through an irritating effect

A detailed comment on the gallstone ileus is outside the scope of this paper Only it should be stressed that no fistula from the gallbladder to the intestinal tract could be disclosed On the other side several cases have been reported (*Murphy* 1910) in which gallstones of very great dimensions have passed through the sphincter

SUMMARY

Four cases of polyps of the small intestine are reported A total of twenty three polyps were revealed Among ten adenomatous polyps four of sessile type showed convincing malignant changes One disclosed invasion of the muscularis mucosae three showed a high degree of cellular anaplasia and several atypical mitoses

One polyp of hamartogenous origin is mentioned Furthermore twelve inflammatory polyps with an appearance earlier designated as pyogenic granulomas are reported These polyps together with some of the adenomas were found in a patient with iliac gallstone obstruction

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Acta path microbiol scandinav 75 257-269 1969

The University Institute of Pathologic Anatomy (Head O Torgersen) Laboratory of Electron Microscopy (Head T Hovig) Rikshospitalet Oslo Norway

THE PERMEABILITY OF MESOTHELIUM TO HORSERADISH PEROXIDASE

A Light and Electron Microscopic Study with Special Reference to the Morphology of Pericardial Mast Cells

By

TROND KLUGE

Received 29 xii 68

The value of horseradish peroxidase as a tracer in studies on protein absorption is generally acknowledged It has proven useful in light and electron microscopic studies on kidney tubules (4 12) on capillary endothelium and on mesothelium of serous membranes Recent experiments have demonstrated absorption of peroxidase along the intercellular spaces in cardiac and muscle capillaries (5) and in pericardial (9) and peritoneal (3) mesothelium These observations strongly suggest that the intercellular clefts represent the morphological equivalent to the "small pore system (10) proposed to explain the absorption of ions and small molecules

It should be considered however whether the peroxidase itself might have an influence upon the permeability of the tissues in question Theoretically the peroxidase molecules may directly or indirectly increase the permeability of the intercellular spaces

In recent publications (3 5 9) it has been stated that such an influence is unlikely as judged from electron micrographs On the other hand peroxidase is known to increase the permeability of venules in different tissues (2) This effect is mediated by histamine and serotonin and can be inhibited by pretreatment with specific antagonists The increase in permeability is accompanied by extensive degranulation of mast cells which are believed to be the source of histamine and serotonin (2)

The purpose of the present study was to further examine whether horseradish peroxidase in usual tracer concentrations does alter the permeability of pericardial mesothelium In particular it was attempted to determine whether application of peroxidase to the mesothelial surface had any influence upon the mast cells in the submesothelial tissue

MATERIALS AND METHODS

Solutions for Injection

a) Horseradish peroxidase Sigma type II (Sigma Chemical Co St Louis Missouri U S A) 0 75 per cent in Ringer s olution
b) 3-3 diaminobenzidine tetrahydrochloride (Sigma Chemical Co) 0 05 per cent in Tris HCl buffer at pH 7 6
c) Evans blue 1 per cent in normal saline
d) Ringer s solution

Experimental Procedure

Adult male and female rats of a local strain weighing 250-3 0 g were anaesthetized by administration of ether/alcohol (2 1) on an open mask or by endotracheal anaesthesia (8) Pericardial injections were done under direct vision (9) while pleural and peritoneal injections were performed percutaneously with a blunt needle

A *Experiments with Evans Blue*

Injections into the serous cavities of different animals were made after the following schedule

Pericardium 0 2 cc of Evans blue and 0 3 cc of peroxidase
Controls 0 2 cc of Evans blue 0 3 cc of Ringer s solution
Pleura 0 5 cc of Evans blue and 0 5 cc of peroxidase into the *left* pleural cavity
Controls (same animal) 0 5 cc of Evans blue and 0 5 cc of Ringer s solution into the *right* pleural cavity
Peritoneum 1 cc of Evans blue and 1 cc of peroxidase
Controls 1 cc of Evans blue and 1 cc of Ringer s solution

The animals were sacrificed 15 30 and 60 minutes following injection The serous cavity was gently flushed with Ringer s solution and the fluid removed The specimens were fixed in 10 per cent formaldehyde cleared with glycerol examined and photographed

B *Specimens for Microscopical Examinations*

0 3 cc of the peroxidase solution was injected into the pericardial cavity and fixation of the tissue was started from 5 to 60 minutes later Initial fixation was performed with 2 5 per cent glutaraldehyde in 0 1 M phosphate buffer Thin slices were removed from the parietal and visceral pericardium and kept in glutaraldehyde for 2 3 hours at 4 C They were washed overnight in 0 1 M phosphate buffer with 5 per cent sucrose and then incubated for 5 minutes in the diaminobenzidine solution The specimens were then washed in 3 changes of distilled water postfixed for 2 hours in osmium tetroxide and embedded in Epon 812

For *light microscopy* sections were cut 0 5-1 mμ thick vertical to the pericardial

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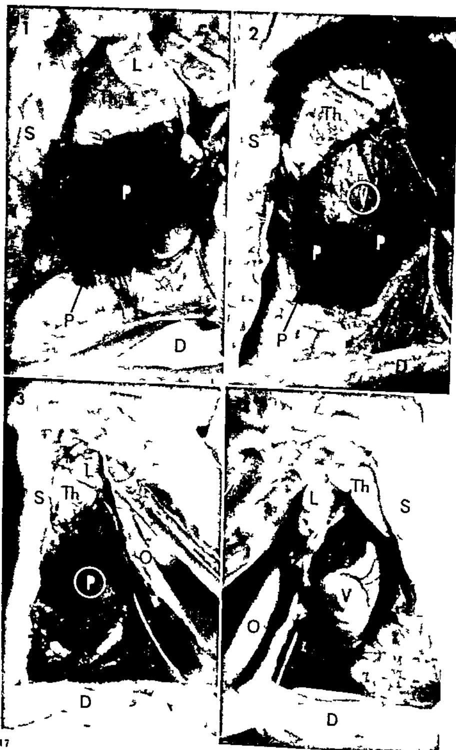
Figs 1-4

Fig 1 View of anterior mediastinum 15 minutes after injection of Evans blue and peroxidase into the pericardial cavity The parietal pericardium (P) has absorbed some of the dye and is stained *No staining of sternum (S) thymus* (Th) mediastinal lymph node (L) and diaphragm (D)

Fig 2 Control animal 15 minutes after injection of Evans blue and Ringer s solution The parietal pericardium (P) shows the same degree of staining as in Fig 1 A circular portion of the parietal pericardium has been excised to show the visceral surface (V) which is unstained

Fig 3 Peroxidase treated animal 1 hour after application There is heavy staining of the parietal pericardium (P) and adjacent areas of the anterior mediastinum including a lymph node (L) Sternum (S) Thymus (Th) Oesophagus (O) Diaphragm (D)

Fig 4 Same animal viewed from the opposite side of the mediastinum The parietal pericardium has been removed The visceral pericardium (V) covering the heart appears unstained


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surface on a Huxley microtome The sections were mounted on glass slides and stained with alkaline toluidine blue at 60 C. They were examined in a *Leitz ultra* microscope equipped with a Photomatic camera

For electron microscopy ultrathin sections were cut from the same blocks with Huxley or LKB microtomes stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop I at initial *magnifications up to 24 000*

RESULTS

The experiments were well tolerated by all animals and toxic reactions were *not observed In particular* there were no signs of circulatory disturbances *suggesting a sudden release of histamine and/or* serotonin

A *Experiments with Evans Blue*

Fifteen minutes after the dye injection a bluish colour was present in the parietal pericardium (Fig 1) while the visceral surface was unstained (Fig 2) The localization and degree of staining seemed identical in the peroxidase treated specimens and in the controls (Figs 1 and 2)

At 30 and 60 minutes the blue colour of the parietal membrane was heavier than at 15 minutes and adjacent parts of the anterior mediastinum were also stained (Fig 3) The visceral surface *did not* show microscopic evidence of dye absorption (Fig 4) Again no difference was observed between the animals exposed to peroxidase solution and *the control animals*

In the corresponding experiments on pleural and peritoneal absorption of Evans blue the dye absorption did not seem to be influenced by the presence of peroxidase As observed in the pericardium staining was far more pronounced in the parietal than in the visceral membranes

B *Light Microscopical Examinations*

Several mast cells were present in the submesothelial area of the pericardium By toluidine blue staining their granules were easily discernible (Fig 5) Specimens from *peroxidase treated animals did* not display any alterations of mast cell morphology (Figs 5 and 6) The cell membrane was well preserved and there were no signs of

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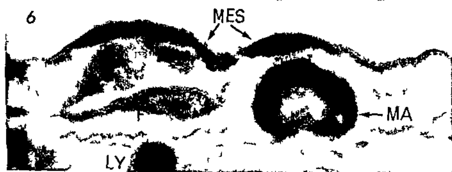
Figs 5–7

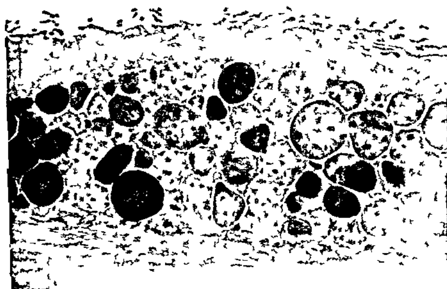
Fig 5 Mast cell (MA) in *pericardial mesothelium 15 minutes after* application of peroxidase The granules are well preserved Toluidine blue staining × 900

Fig 6 1 hour after peroxidase injection *Still normal appearance of mast cell* (MA) The mesothelium (MES) displays numerous microvilli Lymphocytes (Ly) and fibroblasts (*F*) are present *in the submesothelial area* Toluidine blue × 1080

Fig 7 Electron micrograph of the *mast cell in Fig 6* The cell is well preserved and contains numerous granules of normal appearance × 12 000


5
MA


6
MES
MA
F
LY


7

surface on a Huxley microtome The sections were mounted on glass slides and stained with alkaline toluidine blue at 60 C They were examined in a Leitz ultramicroscope equipped with a Photomatic camera

For electron microscopy ultrathin sections were cut from the same blocks with Huxley or LKB microtomes stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop I at initial magnifications up to 24 000

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derangement or disruption of the granules This observation pertained to all time intervals

C Electron Microscopic Examinations

Several mast cells with characteristic large granules were localized close to the mesothelial basement membrane (Figs 7–9) In peroxidase treated tissue the mast cells were surrounded by heavy amounts of peroxidase reaction product (Figs 8 and 9) On some occasions a mast cell was lying immediately underneath an intercellular space which was filled with peroxidase (Figs 8 and 16)

The morphology of the mast cells appeared normal with satisfactory preservation of cell membrane and granules (Figs 7 9 and 16) In particular there was no swelling or disruption of the granules The appearance of the mast cells did not differ from one time interval to another regardless of the amount of reaction product present in the submesothelial area (Figs 9 and 16) Hence the electron micrographs seemed to verify the impression obtained from the light microscopical studies

The appearance of the cell junctions and intercellular spaces was quite variable with tortuous spaces and overlapping of neighbouring cells Closed and open segments were observed along the same cell junction (Fig 10) In some areas a quintuplelayered junction was present thus indicating a fusion of adjacent unit membranes In others a gap was apparent between opposing cells and the junctions could not be considered as tight (Figs 13 and 14)

These observations were valid for untreated animals as well as for those subjected to peroxidase injection The average diameter of the cell junctions did not appear larger in peroxidase treated mesothelium than in the control specimens In the former however the discrimination of unit membranes was often difficult due to the high electron density of the reaction product (Figs 11 and 12) When absorption of peroxidase was at its maximum all intercellular spaces and communicating vacuoles were heavily stained with reaction product (Figs 8 and 15)

DISCUSSION

Among the various types of horseradish peroxidase type II is reported to exert the strongest effect upon venule permeability (2) The cor

Figs 8 and 9

Parietal pericardium 30 minutes after application of peroxidase Heavy staining of intercellular space (IS) and of communicating vacuoles (V) Large amounts of peroxidase reaction product (PPP) are present also in the submesothelial area A mast cell with normal granules (CR) is situated right underneath the intercellular space Fig 8 × 18 000 Fig 9 × 30 000

centration employed was of the usual strength for protein tracing and the amounts were sufficient to give a heavy staining of the intercellular spaces (Figs 8 and 15) Nevertheless the animals showed no signs of generalized vascular injury indicating a release of histamine and/or serotonin Such reactions probably require intravenous administration and doses of peroxidase larger than the ones used in our experiments

Following injections with Evans blue no difference in dye absorption was observed This pertained to the time interval of 15 minutes when staining was weak as well as to the intervals of 30 and 60 minutes when staining was far more heavy Accordingly the results speak against a profound influence of peroxidase upon mesothelial absorption under the present experimental conditions

In the experiments on pleural absorption each animal served as its own control hence obviating the influence of individual variations Furthermore the diaphragm which was used in the evaluation of both pleural and peritoneal staining has a particularly large absorption capacity (1) Finally if the presence of peroxidase had any bearing on Evans blue absorption one would expect a clearly visible difference and not merely a subtle change that might escape detection

There are principally two ways in which peroxidase may exert an influence upon mesothelial permeability

1 By acting directly on the cell junctions
2 Indirectly by releasing histamine and/or serotonin from mast cells

As judged from the light microscopical studies the submesothelial mast cells were not affected by the application of peroxidase and disruption of granules was not suggested This observation was supported by the electron microscopic studies which showed satisfactory preservation of mast cell membranes and granules at all time intervals (Figs 7 8 and 16) At the same time there was heavy labelling of the intercellular spaces immediately subjacent to the mast cells with large amounts of reaction product in the submesothelial area As judged from the micrographs the pericardial mast cells at 30 45 and 60 minutes were literally soaked in peroxidase and still presented a normal morphology

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Figs 10 12

Fig 10 Closed and open segments of intercellular space (IS) in pericardial mesothelium (see text) The closed segments display a central dark line (arrow) which indicates a fusion of adjacent unit membranes × 45 000

Fig 11 Parietal pericardium 15 minutes after injection Small amounts of reaction product are present in the right portion of the intercellular space (IS) The junction (arrow) has a central dark line and appears closed × 30 000

Fig 12 Visceral surface 45 minutes The junction (arrow) seems ers narrow or even closed Peroxidase filled vesicles (V) probably communicate with intercellular space (IS) at another plane of section Nucleus (N) × 45 000



15

16

IS

IS

V

V

V

GR

SA

Mast cells are considered extremely fragile and difficult to preserve (11 a o) In the present work their normal appearance indicates that mast cell damage is not induced by peroxidase in the employed concentrations The lack of degranulation speaks against a local release of histamine and serotonin and indirectly suggests that the permeability of pericardial mesothelium is not increased by such a mechanism

This observation does not agree with the findings by *Cotran & Karnovsky* (2) who report on massive degranulation of mast cells following peritoneal administration of peroxidase This difference is hard to explain It seems unlikely that submesothelial mast cells in pericardial and peritoneal tissue react differently to peroxidase Recent experiments however indicate that certain rat strains are genetically resistant to the permeability increasing effects of peroxidase (6)

In peroxidase treated mesothelium as well as in the control specimens the intercellular spaces and junctions presented a very variable morphology As pointed out by *Karnovsky* (5) the concept of tight and impermeable cell junctions must be revised to a great extent With possible exception for cerebral capillaries the cell junctions in endothelia (5) and mesothelia (3 9) may be open as well as closed under normal circumstances

In the present and in a previous study (9) there was no apparent difference in the average diameter of the junctions in peroxidase treated tissue as compared to that of the control specimens Some junctions appeared closed with a central black line indicating a fusion of the external leaflets of adjacent unit membranes while others showed no such fusion The number and dimensions of closed and open junctions did not seem to be influenced by the absorption of peroxidase

It should be pointed out however that caution must be taken when the morphology of the junctions is evaluated since the electron density of the peroxidase reaction product makes it difficult to determine the fusion or no fusion of unit membranes But as already mentioned if the peroxidase exerted a marked influence upon mesothelial junctions one would expect the morphological equivalents to be easily discernible with the present technique

From the above reasons it is considered unlikely that horseradish

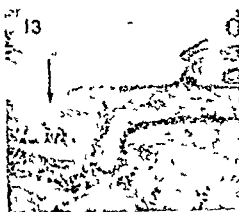
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Figs 13–16

Figs 13 and 14 Apparently no fusion of adjacent cell membranes The junct (arrow) seem to be open Per xida e reaction product is present al n t intercellular clefts Fig 13 from parietal surface at 15 minute 4 f Fig 14 from visceral surfac at 45 minute × 45 000

Fig 15 Parietal mesothelium, 4 minut M ximum absorption of perox heavy taining of the intercellular space (IS) adjacent vacuole n the sul mesothelial area (SA) × 30 000

Fig 16 Parietal mesothelium 1 h ur Ma ked staining of intercellula ce (IS) A sul mesothelial mast cell sh w go d preservation and norm l granules (GR) × 30 000





16

15





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Institute of Forensic Medicine (Head: P. Geertinger, M.D.), Gothenburg, Sweden

PARATHYROID ABNORMALITIES

A Histological Study with Special Reference to Sudden Unexpected Death in Infancy

By

PREBEN GEERTINGER

Received 19.vii.68

In a previous study of sudden unexpected death in infancy (SUD, cot death) evidence was submitted to support the hypothesis that most of these deaths are associated with a congenital incomplete development of the parathyroids (*Geertinger* 1967, 1968). Fusion of parathyroid and thymus tissue at the normal site of the parathyroids was frequently found in the histological serial sections. Furthermore, the number of demonstrable parathyroid glands was greatly reduced.

The technique applied however did not permit an exact evaluation of the number of glands present in each case, neither could the parathyroids found be distinguished as either parathyroid III or IV.

The present examination comprises 17 consecutive cases of typical SUD (age 1-12 months). Histological examination of all organs was routine. In all cases the cause of death remained obscure. In this report only the results of the histological examination of the parathyroids are presented.

TECHNIQUE

The whole parathyroid bearing area from the root of the tongue to the upper mediastinum, including the upper third of the thymus, was fixed as a whole and embedded in paraffin in several orientated parts. About 1000 histological sections (7 my thick and every section mounted) from each infant were cut.

RESULTS

The results are tabulated below (Table I). Fusion of parathyroid and thymus tissue at the normal site of the parathyroids, at or very close to the thyroid capsule, was a very frequent finding. The fusion was complete in all cases, i.e. with common reticulum and vessels in the border zone of the two tissues and lack of fibrous separation. Fusion was only accepted as such when the findings were clearly convincing: the epithelial cells of parathyroid invading the thymic medulla and sometimes even the Hassall's corpuscles. When parathyroid and thymus tissue occurred simultaneously in the sections, either separated by space or by a fibrous capsule, fusion always occurred farther on in the

TABLE 1

The Parathyroids in 17 Cases of Typical SUD (Cot Death)
Histological serial sections of the neck and mediastinum

Autopsy No	Age in months	Sex	Upper Parathyroid (IV) right	Upper Parathyroid (IV) left	Lower Parathyroid (III) right	Lower Parathyroid (III) left
1/66	2	♂	absent	absent	fusion	fusion
15/66	2	♂	absent	absent	absent	fusion
38/66	2	♂	normal	absent	fusion	absent
465/66	2	♀	normal	normal	fusion	fusion
289/66	12	♀	absent	absent	normal	fusion
465/66	4	♀	normal	normal	fusion	fusion
A94/66	4	♂	absent	absent	fusion	fusion
1131/66	2	♀	absent	absent	fusion	fusion
1506/66	2	♂	absent	normal	fusion	fusion
102/67	3	♂	absent	normal	absent	fusion
422/67	1	♀	absent	absent	normal	normal
426/67	3	♀	absent	normal	normal	normal
645/67	2	♂	absent	absent	normal	normal
677/67	3	♂	normal	normal	absent	normal
724/67	7	♀	normal	normal	normal	fusion
141/68	7	♀	normal	normal	fusion	fusion
153/68	2	♂	normal	absent	fusion	fusion

TABLE 2

The Parathyroids in 17 Cases of Typical SUD (Cot Death)
Histological serial sections

	Upper Parathyroid (IV) right	Upper Parathyroid (IV) left	Lower Parathyroid (III) right	Lower Parathyroid (III) left
Expected number of parathyroids	(17)	(17)	(17)	(17)
Parathyroids found				
Normal glands	7	8	5	4
Fusion with thymus tissue	0	0	9	12
Parathyroids absent	10	9	3	1

serial sections The technique applied in the present series permitted a follow up of every bit of parathyroid and thymus tissue Normally the parathyroids are four in number (*Gilmour* 1938) In the present series only 24 normal glands out of 68 possible were found (average 1 4 per infant) If the histologically abnormal (fused) glands are taken into account a total of 44 glands were found (average 2 7 per infant) It will be seen (Table 2) that *absence* of the parathyroids is clearly correlated to the upper glands (parathyroid IV) On the other hand *fusion* with thymus tissue is correlated to the lower glands (parathyroid III) only

Parathyroid tissue was never found in the thymus organ in the mediastinum Extensions of the thymus organ to the normal sites of the parathyroids were in no case demonstrable

RESUME AND DISCUSSION

Histological serial sections of the whole parathyroid bearing area were made in 17 consecutive cases of typical cot death (SUD)

A high frequency of absence of the upper glands (parathyroid IV) and of histological abnormalities (*fusion with thymus tissue*) of the lower glands (parathyroid III) was demonstrated

A control series of infants (aged 1–12 months) with definitely established causes of death (accidents homicides) is not yet available but is eagerly sought Fusion of parathyroid and thymus tissue is claimed to occur occasionally in normal infants (*Gilmour* 1941) but the frequency of this phenomenon still remains to be elucidated If the fusion of the *lower parathyroids* with thymus tissue which occurred with such significant frequency in the present series is not pathognomonic of cot death (SUD) it is certainly *normal* human anatomy rather than a relatively rare variation as *Gilmour* (1941) seemed to believe A *functional* difference between the upper and lower pairs of parathyroids parallel with the histological differences should be considered The discovery of calcitonin (*Copp & Cheney* 1962) and the still lingering uncertainty as to the site of production of this second parathyroid hormone invite reconsideration of the anatomy and histology of these glands and their relation to thymus tissue

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University of Louisville School of Medicine Department of Pathology and Radiology
Louisville Kentucky U S A

COLITIS CYSTICA PROFUNDA

By

PER HENRIK BECHER CARSTENS and RAUL GONZALEZ

Received 5 viii 68

Two types of cystic enlargement of the mucous glands in the lower gastro intestinal tract have been described superficial and deep The former has been designated colitis cystica superficialis and is closely related to pellagra (*Denton* 1925) The latter is colitis cystica profunda and has been associated with chronic dysentery and chronic ulcerative lesions of the colon

Colitis cystica profunda was first described by *Stark* in 1766 *Virchow* in 1863 described a similar lesion in a 15 year old male who died of chronic dysentery He coined the term colitis cystica polyposa because the polyps were more solid than cystic Recently several cases have been reported in the American literature with special emphasis on the clinical picture morbid anatomy the resemblance to mucinous adenocarcinoma of the colon and theories on the aetiology

The purpose of this paper is to report a single case with some unusual features and to discuss the literature and aetiology

CASE REPORT

The patient (L G H 499 065) was a 44 year old Caucasian female who had recently received treatment for delirium tremens She recovered quickly without complications Upon discharge from the hospital she was sent to a mental hospital There she complained of being weak It was not possible at that time to get a history from the patient although it was mentioned that she had had rectal bleeding for the last 6 months The only positive finding on physical examination was a prolapse of the rectum which was very tender but reducible digitally Slight abdominal distension and generalized abdominal tenderness were noted The rest of the physical examination was not remarkable

Proctoscopy and sigmoidoscopy revealed the mucosa to have a granular appearance with areas of haemorrhage A biopsy of the rectum showed chronic inflammation and ulceration of the mucosa A barium enema was performed and the distal colon up to the transverse colon showed spasticity and polypoid lesions There was also evidence of sinuses and opacification by the barium in small cysts The transverse colon ascending colon and caecum showed no abnormalities (Fig 1)

Laboratory test were not remarkable The patient's rectal prolapse was t[illegible] with baths and suppositories She was given enemas and expelled a con[illegible] quantity of faeces and mucus Later the patient developed diarrhea with [illegible] mucus The haemoglobin fell from 13 8 to 9 2 grams A blood transfusion [illegible] given She was transferred to Louisville General Hospital four days later for a laparotomy At operation a perforation was found in the descending colon close to the sigmoid This was [illegible] and exteriorized Twenty four hours after surgery the patient expired in pulmonary distress



Fig 1

Barium enema showing loss of haustral pattern together with small cysts situated parallel to the lumen

Autopsy Examination

The exteriorized perforation was closed by silk sutures There was localized peritonitis for a distance of 5 centimeters on each side of the perforation The mesenteric arteries showed only mild atherosclerosis and were patent throughout A few enlarged lymph nodes were found scattered in the mesentery The muscularis of the descending colon and rectum appeared diffusely thickened from the splenic flexure

When the colon was opened the mucosa had a cobblestone appearance with multiple pseudopolyps up to 1 centimeter in diameter interspersed by deep narrow ulcers The lumen contained formed faeces covered by a minimal amount of mucus On sectioning none of the pseudopolyps contained grossly visible cysts No mucus could be expressed from the polyps into the lumen The ulcerated area was sharply demarcated 10 centimeters distal to the splenic flexure blending into normal but distended colon at this point There was no demarcation distally No normal appearing mucosa was found in the descending colon sigmoid or rectum The only other findings of significance were emboli in the arteries of the right lung and extensive bronchopneumonia (Fig 2)

All the sections from the involved colon showed the same picture of

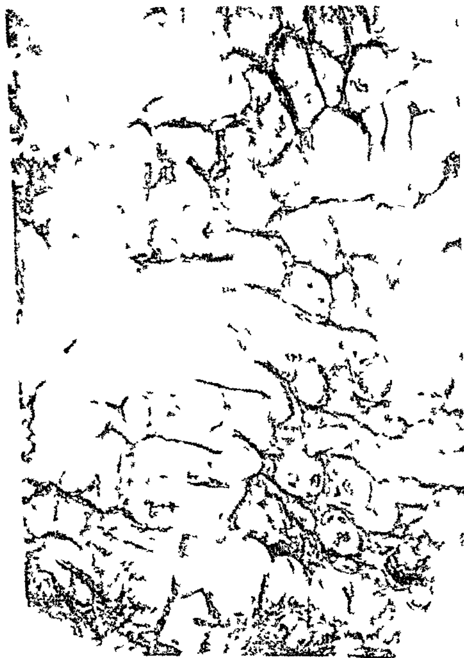




Fig 3

Photomicrograph of sigmoid colon The mucosa at top of photo shows congestion and inflammatory changes Below the muscularis mucosa several cysts lined by columnar cells

deep ulcers many going down to the muscularis propria alternating with islands of pseudopolyps Under these pseudopolyps in the oedematous submucosa below the muscularis mucosa a number of dilated and enlarged glands varying in size up to 5 millimeters in diameter were found These glands contained purulent exudate and P A S positive material On serial sectioning many of the glands were found to be continuous with the overlying mucosa The glands described above were found only in the sections from the descending colon and sigmoid The lining cells of the glands were usually columnar but in some areas cuboidal cells were seen In other areas the lining cells were completely absent (Fig 3)

The submucosa was oedematous containing large numbers of lymphoid follicles and mixed type of inflammatory cells together with red blood cells In some areas a few giant cells of the foreign body type were present No granulomas were noted The muscularis propria showed a great amount of hypertrophy and oedema The ganglion cells of Auerbach s plexus showed degenerative changes In sections close to the perforated area there was acute peritonitis No parasites were seen in any of the sections The mesenteric arteries showed only mild to moderate atherosclerosis and the lymph nodes reactive hyperplasia

DISCUSSION

Since 1951 35 cases of colitis cystica profunda have been reported in the English literature Between the first description in 1766 and these recent reports only a few cases have been mentioned (*Manson Bahr & Gregg* 1925 *Woodward* 1879) These authors describe the cysts and their relationship with dysentery There is a special reason to draw attention to Woodward s work He goes into very elaborate descrip

TABLE 1

Colitis Cystica Profunda Diffuse Type

Authors	Goodall H B & Sinclair I S R.		Wayte D M & Helwig E B
Number of cases	2		5
Sex	Male	Female	All men
Age	43	24	4–68 mean age 3[illegible]
Initial symptoms	Weakness weight loss and diarrhea	Rectal passage of blood and mucus	Intermittent rectal bl[illegible]
Associated disease	——	Chronic ulcerative colitis	Two Ulcerative colitis Two Multiple polyps in the colon One Chronic dysentery

TABLE

Colitis Cystica Profunda

Authors	*Thompson H. R.*	*Castlemann B.*	*Allen M. S.*		
Number of cases	1	1	3		
Sex	Male	Male	Male	Female	Male
Age	27	37	33	38	18
Initial symptoms	Abdominal pain and rectal passage of blood and mucus	Abdominal pain and diarrhea	Abdominal pain and diarrhea	Red blood on stool	Rectal passage of blood and mucus
Associated disease	7 years previously treated with diagnosis of ulcerative colitis	——	——	——	Hemorrhoids and fissure

tions of the lesion and presents a very beautiful colour plate of the colon in one of the three reported cases. Woodward also offers an explanation of the lesion: "In catarrhal inflammation of the intestinal mucous membrane the softened parenchyma of the swollen solitary follicles offers a suitable nidus for the peculiar cyst-like development of the glands of Lieberkuhn, which elsewhere is prevented from going beyond a very moderate degree by the resistance offered by the muscle of Bruecke; and whether the cystic transformation of the hypertrophic gland tubules proceeds to a moderate or an extreme degree, the parenchyma of the follicles is the site in which the cystic development occurs." In 1951 *Thompson* described a single case with the diagnosis of chronic non-specific ulceration associated with inclusion cysts. In 1957 *Goodall & Sinclair* reported two cases. One of their patients had ulcerative colitis. The authors believed that the cysts were the result of trapped regenerating surface epithelium in the submucosa caused by lymphoid aggregates with abscess formation and subsequent destruction of the muscularis mucosa.

In 1963 and again in 1966 *Castleman* reported a single case which had a great loss of protein from the lesion and used the term mucocele of the bowel. On x-ray examination this patient showed minor changes in the rectal mucosa pattern over a period of several years. More recent films showed nodular polypoid masses in the rectum and in some places outpouchings of the barium in a spicular fashion. A con-

2
Localized Type

Epstein S E et al				*Wayte D M & Helwig E B*	
4				19	
Female	Male	Female	Female	10 male	9 female
55	63	36	15	20–51 mean 28	20–52 mean 34
Rectal bleeding	Polyps and diarrhea	Rectal prolapse and bleeding	Rectal passage of blood and mucus	Mass Rectal bleeding Mucus discharge	
——	All three patients had their symptoms for several years			All 19 patients had histologic evidence of an associated chronic proctitis	

genital defect in the muscularis mucosa was favoured in consideration of the aetiology

Allen (1966) described three young adults with similar lesions and offered a hamartomatous theory of pathogenesis *Epstein* (1966) who presented 4 cases assumed a defect in the muscularis mucosa caused either by inflammatory damage or congenital defect Finally *Wayte & Helwig* in 1967 reported 24 cases from the files of the Armed Forces Institute of Pathology and were the first to divide the lesions into two groups namely a diffuse type and a localized type in which cysts were confined to a distinct area of the rectum Among these 24 cases 19 were localized and all these showed evidence of associated chronic proctitis Among the five in the diffuse group two were associated with chronic ulcerative colitis and one patient had chronic dysentery *Salmonella Newport* These authors considered the cysts to be a result of the extension of surface mucosa along granulation tissue tract created through the muscularis mucosa

Tables 1 and 2 show the 35 known cases divided into diffuse and localized type with information of age sex initial symptoms and associated diseases In the diffuse group is a predominance of young men and the relationship to inflammatory diseases is constant The most common initial symptom is intermittent rectal bleeding In the localized group the sex ratio is nearly 1—1 and the association with inflammatory diseases in the rectum except for Wayte and Helwig s

group is less obvious Here the main initial symptom except for rectal bleeding is the finding of a mass

Each one of the following authors had one case with the diagnosis of malignant tumour *Allen Epstein Goodall & Sinclair, Wayte & Helwig* Special attention should be drawn to the fact that although the finding of glandular elements in the submucosa may cause the diagnosis of adenocarcinoma to be entertained there is a complete absence of any cytological features suggesting cancer

This case belongs to the diffuse group and was associated with subacute ulcerative colitis No large cysts were noted either grossly or microscopically A reason for this may be that the ulcers in the colon were large and the corresponding defects in the muscularis mucosa wide allowing the mucus formed in the trapped cysts to escape freely into the bowel lumen and therefore preventing enlargement of the glands The x ray however showed the above mentioned spicular effect and suggested the diagnosis We believe that the cysts in our case were caused by defects in the muscularis mucosa produced by inflammatory changes which allowed regenerating mucosa to form cysts in the submucosa This theory has recently gained support by experimental work by *Brynjulfsson & Haley* (1967) who grafted segments of small intestine to the abdominal wall of rats and after 5 to 19 months were able to demonstrate lesions similar to colitis cystica profunda in 11 of 12 animals

Finally we would like to suggest different aetiologic factors for the diffuse and localized form of colitis cystica profunda The diffuse type originates from colitis which creates defects in the muscularis mucosa and acts like a trap for the regenerating mucosa In the localized type however the cystic dilatation may be attributed to a mild chronic proctitis which will produce enlargement of congenital mucosal cysts in this location These cysts or vacuoles have been described by the embryologist *F P Johnson* (1913 1914) as originating during the lumen formation of the intestines in certain locations such as the oesophogastric junction second portion of the duodenum vermiform appendix caecum and rectum The vacuoles usually disappear about the time of birth but may persist and could be the initial weak spot for the development of the localized form of colitis cystica profunda

SUMMARY

A case of *colitis cystica profunda* with some unusual features is presented

The previously reported cases in the literature are discussed with special emphasis on aetiology The division of colitis cystica profunda into a diffuse and localized type first suggested by *Wayte & Helwig* has been applied to the 35 reported cases

Finally we suggest two different aetiologies for the diffuse and loca

lized form The diffuse form being caused by colitis and the localized form by congenital mucosal cysts with superimposed infection

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Acta path microbiol scandinav 75, 282-290, 1969

The University Department of Pathology, General Hospital, Malmö, Sweden

ALVEOLAR RHABDOMYOSARCOMA

By

PAWEŁ MIKULOWSKI and THORBJÖRN BERGE

Received 6.viii.68

Alveolar rhabdomyosarcoma was first described by *Riopelle & Thériault* in 1956. *Horn & Enterline* (1958) studied 39 cases of rhabdomyosarcoma and found a distinct alveolar pattern in 8 of them. In another paper (*Enterline & Horn* 1958) they gave a detailed description of the characteristic clinical and pathological features of this type of tumour. Since then only a few cases have been reported (*Goguel et al.* 1962, *Mikulowski* 1962, *Valdes Ruiz et al.* 1962, *Porterfield & Zimmerman* 1962). The tumour may be more common than widely believed. In an article on sarcomas of the head and neck *Bardwil et al.* (1964) described 45 cases of rhabdomyosarcoma, 20 of which were classified as alveolar but were not described in detail.

Alveolar rhabdomyosarcoma has often been confused with one or another of a wide variety of neoplasms including carcinoma, melanoma and juvenile melanoma, paraganglioma, reticulum cell sarcoma, neuroepithelioma, Ewing's sarcoma, mesothelioma and different types of angiosarcoma (*Enterline & Horn* 1958, *Riopelle & Thériault* 1956). The tumour, however, is a distinct clinicopathological entity, differing biologically from many of the tumours mentioned above, and adequate treatment requires a correct diagnosis. Since no report of the tumour could be traced in the Scandinavian literature, 3 cases seen at our department are described below.

CASE REPORTS

Case 1. C. B. J.

A 34 year old woman was expecting her second child. At routine control at the prenatal clinic in the 5th month a cyst like tumour, 7 × 7 cm, was found in the right breast. Frozen section diagnosis (PAD 10737/6): Medullary carcinoma. Immediate radical surgery revealed residual tumour but no involvement of the lymph nodes. One month after delivery (at full term) a local recurrence was treated surgically as well as radiologically (x-ray 2 800 R). Bilateral oophorectomy was performed and androgen treatment was given as routine procedures in the treatment of carcinoma of the breast (no metastases to the ovaries). Later the patient was given radiation (2 100 R) of the neck because of metastases and of the cervical and lumbar spine (2 400 R) because of pain. Abdominal metastases were treated with cobolt radiation (4 000 rad). The patient was also treated for 7 weeks with a cytostatic (Sendoxan® 50 mg × 2). Death occurred 12 months after the first operation.

Autopsy (11.9/67) revealed generalized tumour growth (lungs, heart, liver, kidneys, adrenals, skeleton, dura mater of the brain and of the spinal cord, oesophagus

stomach peritoneum vagina and mediastinal retroperitoneal supraclavicular axillar and inguinal lymph nodes)

The findings made at histological examination of necropsy specimens and re examination of the operative specimens were compatible with alveolar rhabdomyo sarcoma There was no clear cross striation but many of the tumour cells contained myofibril like structures The mitotic frequency was high

Case 2 T D N

A 3 year old boy had a lump in the right mandibular angle The bony tissue was unaffected but the tumour which had been noticed two weeks previously had in filtrated the cheek and the soft palate Radical surgery was impossible and after biopsy (PAD 1606/63 diagnosis Unspecified malignant tumour) he was treated with a cyt stat c (Sendoxan® t tally 1 850 mg) and x ray irradiation (3 500 R) of two fields in the right parotid region A smaller area in this region was given a further dose of 500 R 3 200 R was applied to a field above the right auricle and 2 100 R to the left parotid region and upper left side of the neck There was no appreciable effect of the treatment and the boy died 8 months later

Autopsy (1036/63) revealed massive local growth of the tumour with infiltration of the tongue and the base of the skull The lungs and the mediastinal lymph nodes contained many metastases

Histologically most of the tumour was rhabdomyosarcoma of ordinary embryonal type but some parts of the primary tumour as well as the metastases contained prominent alveolar structures The mitotic figures were common

Case 3 P S

A 9 year old boy who had had a stiff neck for about 4 weeks An apparently circumscribed tumour 3 × 2 × 2 cm was found subcutaneously in the right mandibular region The bony tissue was unaffected A fine needle biopsy (PAD 10544/64) revealed a malignant growth (The cytological picture is described in the discussion) The tumour was then extirpated and x ray treatment (800 R to two fields in two series) was given Histologically (PAD 10716/64) the tumour was a typical alveolar rhabdomyosarcoma it had no capsule and it had infiltrated adjacent tissues The mitotic frequency was high No sign of tumour 4 years later

DISCUSSION

The true incidence of alveolar rhabdomyosarcoma is not known Our 3 cases were diagnosed among approx 13 000 autopsies and 150 000 surgical biopsies but some cases were probably missed Since the tumour may have been confused with carcinoma and melanoma as well as with different types of sarcoma re examination of all possible cases was impracticable The tumour occurs chiefly in children and in young adults The cases hitherto published are summarized in Table 1

The mean age of the patients is about 15 years it is lower than the corresponding age in cases of pleomorphic rhabdomyosarcoma 50 8 years) and higher than that in cases of sarcoma botryoides and embryonal rhabdomyosarcoma (8 9 years) (*Enterline & Horn* 1958) The sex relation was 19 males and 11 females The primary tumour may occur anywhere but is often seen in a limb or in the orbit The tumours are as a rule indolent They usually originate in muscle tissue but some arise subcutaneously as in our case 3 and possibly also in case 2 The tumour grows rapidly and metastasizes early It is disseminated by the blood stream and the lymphatic circulation metastases are often found in regional lymph nodes in the viscera and in the skeleton Se

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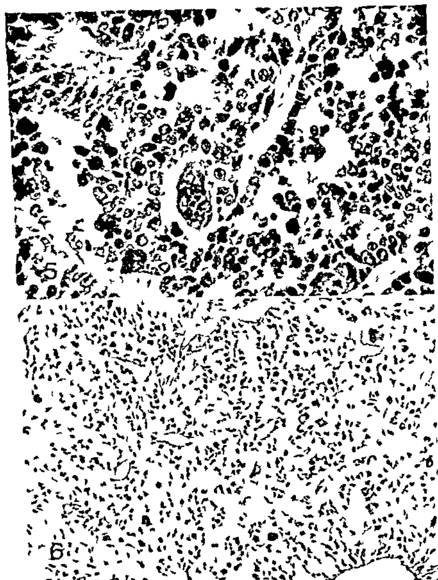
Alveolar rhabdomyosarcoma has often been confused with one or another of a wide variety of neoplasms, including carcinoma, melanoma and juvenile melanoma, paraganglioma, reticulum cell sarcoma, neuroepithelioma, Ewing's sarcoma, mesothelioma and different types of angiosarcoma (*Enterline & Horn* 1958, *Riopelle & Thériault* 1956). The tumour, however, is a distinct clinicopathological entity differing biologically from many of the tumours mentioned above, and adequate treatment requires a correct diagnosis. Since no report of the tumour could be traced in the Scandinavian literature, 3 cases seen at our department are described below.

CASE REPORTS

Case 1. G.B.J.

A 34-year-old woman was expecting her second child. At routine control at the prenatal clinic in the 5th month a cyst-like tumour 7 × 4 cm was found in the right breast. *Frozen section diagnosis (PAD 10737/6.)*: Medullary carcinoma. Immediate radical surgery revealed residual tumour but no involvement of the lymph nodes. One month after delivery (at full term) a local recurrence was treated surgically as well as radiologically (x-ray 2800 R). Bilateral oophorectomy was performed and androgen treatment was given as routine procedures in the treatment of carcinoma of the breast (no metastases to the ovaries). Later the patient was given radiation (2100 R) of the neck because of metastases and of the cervical and lumbar spine (2400 R) because of pain. Abdominal metastases were treated with cobalt radiation (4000 rad). The patient was also treated for 7 weeks with a cytostatic (Sendoxan® 50 mg × 2). Death occurred 12 months after the first operation.

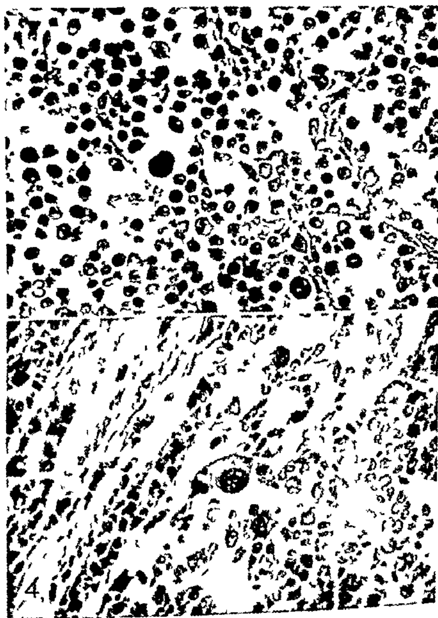
Autopsy (1159/67) revealed generalized tumour growth (lungs, heart, liver, kidneys, adrenals, skeleton, dura mater of the brain and of the spinal cord, oesophagus



Figs 5–6

Fig 5 Detail of alveoli with lining cell and solid formation of floating cells One syncytial cell and a few smaller multinucleated cells (Phosphotungstic acid haematoxylin × 350) (Case 1)

Fig 6 Less prominent al eolar stru ture numerous blood vessels Characterist ll dispersed t pical giant s n tial cells (Pho photungstic acid haematox lin × 350) (Case 2)



Figs 3–4

Fig 3 Al eoll containing floating cells pronounced polymorphism Centrally a strap cell with brightly oxyphilic cytoplasm (Haematoxylin eosin × 350) (Case 3)

Fig 4 Area with general characteristics of rhabdomyosarcoma Such parts are useful in the establishment of a differential diagnosis (Phosphotungstic acid haematoxylin × 350) (Case ?)

The mitotic activity varies widely from case to case It seems not to have any prognostic value

3 The occurrence of giant syncytial tumour cells with multiple often peripherally located atypical nuclei and with abundant oxyphilic cytoplasm *The cells occur in alveolar spaces but are often attached to* fibrous septa They never tend to accumulate in groups The scattered arrangement of syncytial cells gives a characteristic pattern in low magnification (Figs 5 and 6) Syncytial cells are often seen but are not a constant finding The tumour also contains large anaplastic cells with bizarre rounded or lobulated nuclei and with relatively little cytoplasm These cells are less characteristic histologically (though prominent in smears) but are apparently always demonstrable

We feel that these features are important in the histological diagnosis of alveolar rhabdomyosarcoma *There are some further but less con* stant features The cells in one and the same tumour vary *in amount and appearance of the cytoplasm The cytoplasm may be granulated and* often contains myofibril like structures Vacuoles containing PAS posi tive material are common Cross striation has been *described* in about two thirds of all cases usually only after prolonged search No cross striation was seen with certainty in any of our cases Fine needle punc ture of the tumour (case 3) in this series produced an aspirate rich in cells and the smears showed cells of the types described in the histo logical slides The eccentric nuclei were irregular the chromatin was coarse and some of the nuclei contained large nucleoli Giant cells with multiple nuclei or one bizarre lobulated nucleus were easily recognized Mitoses were common (Figs 7 and 8) No cross striation was demon strated *The amount of cytoplasm varied and a perinuclear halo* was usually seen *In many* cells the cytoplasm was vacuolated

It is known that this tumour *originates from skeletal muscle* but yet it is difficult to explain the alveolar pattern According to *Enterline & Horn* (1958) the tumour cells lining the spaces correspond to a syn cytium of rhabdomyoblasts We were however able to recognize dis tinct borders between the tumour cells lining the spaces as well as the floating cells (Masson s trichrome stain) There is however *no* analogy between the alveolar structures and the embryonal features of muscle The tumour gives the impression of inducing the formation *of* septa built up of apparently mature fibrous tissue Such a benign stroma is rare in *malignant* mesenchymal tumours It is also possible that septa *may* have *formed* by local tissue destruction caused by the tumour and by *proliferation* of tumour cells along preserved vessels The giant cells resemble those seen in degenerated muscle cells *e g* in desmoid tumours and congenital torticollis

The clinicopathological features described are *important for a differ* ential diagnosis The presence of multiple nucleated syncytial giant cells helps to exclude other entities such as carcinoma and paraganglioma The presence of floating cells devoid of reticulum fibres *should*

probably be sufficient to exclude angiosarcoma There should be no difficulty in ruling out reticulum cell sarcoma melanoma mesothelioma and malignant synovioma if abundant and well preserved tumour tissue is available Cross striation is decisive but it is not a conditio sine qua non for diagnosing rhabdomyosarcoma

Two of our cases were of special interest As far as we know mammary alveolar rhabdomyosarcoma has never been described Malignant muscle tumours of the breast are very rare but are as a rule not difficult to differentiate histologically from carcinoma Such confusion as happened in our case 1 is however possible in cases of alveolar rhabdomyosarcoma with its peculiar pseudoepithelial appearance It is therefore necessary for the pathologist to be familiar with the condition It appears that the patient in our case 3 is the only one on record who has survived for 4 years without any signs of recurrence or metastasis

SUMMARY

Three cases of alveolar rhabdomyosarcoma are reported In one of the cases a woman 34 years old the tumour was located in the breast As *far as we know this location has not been described before* Another case was a 3 year-old boy with generalized growth of mixed embryonal and alveolar rhabdomyosarcoma The third case was a boy 2 years old who is well and without signs *of tumour 4 years after diagnosis* A detailed description of the pathology is given and the differential diagnosis is discussed Our findings are correlated with literature cases

ADDENDUM

Olufemi Williams et al have recently described electron microscopic investigation of one case of alveolar rhabdomyosarcoma (Brit J Ca 2? 12–18 1968)

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Acta path microbiol scandinav 75 291-295 1969

The Municipal Bacteriological Laboratory Aurora Hospital Helsinki
and the State Serum Institute Helsinki Finland

MARKED CROSS AGGLUTINATION BETWEEN *BRUCELLAE* AND A SUBTYPE OF *YERSINIA ENTEROCOLITICA*

By

P AHVONEN ELLI JANSSON and K AHO

Received 18 vii 68

Recently attention has been paid to *Yersinia enterocolitica* (formerly *Pasteurella X*) as a cause of mesenteric lymphadenitis acute terminal ileitis and erythema nodosum (*Winblad et al* 1966 *Mollaret* 1966 *Ahlehn & Sjostrom* 1967) The taxonomic relationship between *Yersiniae Pasteurellae* and *Francisellae* has not been definitely settled *Yersinia enterocolitica* can be readily differentiated from *Yersinia pseudotuberculosis* (syn *Pasteurella pseudotuberculosis*) by a series of biochemical tests (*Knapp & Thal* 1963 *Mollaret & Chevalier* 1964) Serologically both can be divided into several types (*Knapp* 1959 *Winblad* 1967) Besides strains of *Yersinia enterocolitica* show slight differences from each other in biochemical tests (*Mollaret et al* 1964 1965 *Ahlehn* 1967)

Bovine brucellosis has been eradicated in Finland (*Huhtala* 1963) and for many years at least bacteriologically verified human cases have not been diagnosed A series of human sera containing brucella agglutinins corresponding to 100–1600 IU but suspected to be falsely positive for the above reason had been surveyed for various antibodies with a view to revealing the possible cross reaction basis of this agglutination All the sera were observed to give high agglutination titres with one subtype of *Yersinia enterocolitica* It is the purpose of this communication to describe this cross-agglutination reaction

MATERIAL AND METHODS

Human sera Twenty four human sera containing brucella agglutinins corresp ... to 100 16 0 IU were used in the study Sixteen sera were found among about ... specimens sent in for routine Widal te t Four sera were found amon ab ut ... specimens submitted for toxoplasma antibody studies Four sera der ... patient suspected to have yersinia inf ... on

Rabbit immune sera Rabbits were g ... n two intravenous injection ... and 0 5 ml) one week apart using the following antigens Brucella abortus a t ... used in the agglutination test (tw rabbit *Brucella melitensis* anti en (ne rabbit) *Yersinia enterocolitica* strain ... autoclaved at 120 C for tw hours (one rabbit) and living strain ... 9 (two rabbits) The str ngth of the antigen sus

pensions corresponded to that used in the agglutination tests. Bleedings were made one week after the second injection.

Antigens. *Yersinia enterocolitica* strains representing the four serologically different types isolated from man were obtained from Dr Birgitta Niléhn, General Hospital, Malmö, Sweden. 1) Strain "Winblad" was isolated by Carlsson (*Carlsson et al.* 1964) and represents the most common type of Yersinia enterocolitica found in Sweden. It contains the O antigen III described by *Winblad* (1967) and is referred to in this paper as Yersinia enterocolitica type III. 2) MY 79 was isolated by *Niléhn* in 1966. Its O antigen was not included in the antigen scheme prepared by *Winblad* (1967) and according to his suggestion the strain is referred to here as *Y. enterocolitica* type IX. 3) E 219 was isolated by *Bojsen Møller* in 1961. 4) Albany 33114 (*Bacterium enterocoliticum* Schleifstein & *Coleman* 1943).

Yersinia pseudotuberculosis types I–V were obtained from *Prof. W. Knapp*, Erlangen, Germany.

Brucella abortus strain 544 and *Brucella melitensis* strain 16M (FAO/WHO reference strains) were obtained from Dr. W. J. Brinley Morgan, FAO/WHO Brucellosis Centre, Weybridge, England.

In the case of *Y. enterocolitica* type III an O antigen and an OH antigen were used; in the case of the other *Yersiniae* an OH antigen, all prepared as described by *Winblad et al.* (1966). The brucella antigens were prepared according to *Alton & Jones* (1964). The densities of the antigen suspensions were measured with a Hitachi Perkin Elmer spectrophotometer to give an absorbance of 0.30 at wave length 540 mμ (10 mm cuvette).

Agglutination and absorption tests.—The sera were inactivated at 56° C for 30 minutes. Series of twofold dilutions in saline were made starting with a dilution of 1:20. To 0.25 ml of serum dilution an equal volume of antigen suspension was added and the series were incubated at 52° C overnight. The titration endpoint was recorded as the highest serum dilution giving an agglutination visible to the naked eye. The titres are expressed as reciprocals of serum dilutions.

For the absorption 2 ml of serum dilution 1:20 was mixed with the centrifuged deposit of the antigen (corresponding to 80 ml of the antigen suspension used in the agglutination test) and incubated at 37° C for two hours. If the bacteria were completely sedimented the absorption was repeated. Parallel tests with unabsorbed serum were always done. On each occasion 2–5 sera were examined and the series were read blindly without knowledge of the contents of the tubes.

RESULTS

Eight human sera were tested with four different *Yersinia enterocolitica* types and with *Yersinia pseudotuberculosis* types I–V. They all gave strong reactions with *Y. enterocolitica* type IX while negative reactions were obtained with the others.

Table 1 shows the results of all the 24 patient sera with the *Brucella*

TABLE 1

Titres of 24 Patient Sera with Brucella abortus *and* Yersinia enterocolitica *Type IX Antigens*

Yersinia enterocolitica type IX	Number of sera with titres against Brucella abortus 160	320	640	1280	2560
160	1	–	–	–	–
320	3	3	–	–	–
640	3	3	1	–	–
1280	1	–	6	1	–
2560	–	1	–	–	1

abortus and *Y. enterocolitica* type IX antigens. Titrations with these were always performed on the same occasion. Titres obtained with *Y. enterocolitica* type IX were slightly higher or at the same level but never lower than those obtained with *Brucella abortus*. With the technique employed the International Standard of *Brucella abortus* serum containing 1000 IU/ml gave a titre of 1280 with both antigens.

The majority of the sera were also tested with *Brucella melitensis* antigen. The results closely resembled those obtained with *Brucella abortus*.

TABLE 2

Reduction of Yersinia enterocolitica *Type IX Titre in 24 Patient Sera after Absorption with* Brucella abortus

Agglutination titre before absorption	Titre reduction in twofold dilution steps							
	0	1	2	3	4	5	6	7
160	–	–	–	–	1	–	–	–
320	–	1	2	–	–	3		–
640	–	2	1	–	3	–	1	–
1280	2	–	1	1	1	2	–	1
2560	1	–	–	–	–	1	–	–
	Number of sera							

Reverted to negative

TABLE 3

Results of Cross Absorption with Rabbit Immune Sera against Brucella abortus *and* Yersinia enterocolitica *Type IX*

Immune serum against	Absorbed with	Agglutinin titre	
		Y. enterocol. type IX	*Brucella abortus*
Y. enterocolitica type IX (living antigen)	unabsorbed	2560	1280
	Y. ent. IX	<20	<20
	Br. abortus	1280	<20
Brucella abortus	unabsorbed	2560	2560
	Y. ent. IX	<20	40
	Br. abortus	<20	<20

All the sera were tested with tularaemia antigen: six of these gave titres of 20–40.

To obtain information concerning the infective agent cross absorptions were performed. In each instance no homologous agglutinins were detected after the absorption. The absorption with the yersinia antigen always removed brucella agglutinins. It is seen in Table 2 that the absorption with *Brucella abortus* antigen gave varying results. In 12 sera there was a marked reduction of titre (≧ 2 twofold dilution steps) but some yersinia agglutinins were detected. In six instances there was only insignificant or no titre reduction (0–1 steps), whereas

in six others the absorption with *Brucella abortus* removed all detectable agglutinins against *Y. enterocolitica* type IX.

Table 3 illustrates the cross absorption of two rabbit immune sera. Noteworthy is the very marked titre reduction of *Brucella abortus* immune serum after the absorption with *Y. enterocolitica* type IX. Similar types of results were obtained with the other immune sera.

DISCUSSION

It has been reported that *Brucellae* cross react in agglutination tests with *Francisella tularensis*, *Vibrio cholerae*, *Vibrio fetus* and some *Salmonellae* (*Morse et al.* 1953, *Wundt* 1961). These cross reactions are of minor degree in the sense that the titres with cross reacting antigens are markedly lower than the homologous titres. Marked cross reactions usually reflect a close taxonomic relationship. However, this need not be so, provided that the cross reacting antigen is a good immunogen present in sufficient quantities and suitably located on the surface. This is exemplified by the cross reaction between *Rickettsiae* and certain strains of Proteus.

The cross agglutination between *Brucellae* and *Yersinia enterocolitica* type IX observed in the present work is marked, since the titres with the cross reacting antigen were at the same level as the homologous titres, and the absorption with the cross reacting antigen reduced the homologous titres. This was observed both with sera from patients and with sera prepared in rabbits. The cross absorptions suggested in most instances that the infective agent had been *Yersinia enterocolitica* type IX rather than any brucella, and in no instance did they provide evidence to the contrary.

Based on a series of biochemical tests, type IX closely resembles the other subtypes of *Yersinia enterocolitica* (*Mléhn et al.* 1968). It differs from *Brucellae* in that it grows better on various media as well as anaerobically, is motile at 22° C (but not at 37° C), and produces acid in many of the conventional sugar fermentation tests.

The clinical picture of the disease in the patients has been analysed (*Ahvonen & Sievers* 1968) and found to be compatible with that observed in infections caused by other types of *Y. enterocolitica* (*Winblad et al.* 1966, *Mollaret* 1966). However, due to the diversity of symptoms observed in human brucellosis (*Elberg* 1965), the clinical picture in some cases also fitted in with that of brucellosis.

It is apparent that problems involved in the serological diagnosis of brucellosis arise in areas where infections caused by *Y. enterocolitica* type IX do occur.

SUMMARY

Twenty four human sera containing brucella agglutinins corresponding to 100–1600 IU had agglutination titres of the same level or slightly

higher against Yersinia enterocolitica type IX. Very weak or no reactions were obtained with other yersinia antigens and with tularaemia antigen. Cross absorptions suggested that the infective agent had been *Yersinia enterocolitica* type IX rather than any *Brucella* in the majority of cases. Rabbit antisera against these bacteria gave titres of the same level with both antigens.

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It has been reported that *Brucellae* cross react in agglutination tests with *Francisella tularensis*, *Vibrio cholerae*, *Vibrio fetus* and some *Salmonellae* (*Morse et al.* 1953, *Wundt* 1961). These cross reactions are of minor degree in the sense that the titres with cross reacting antigens are markedly lower than the homologous titres. Marked cross reactions usually reflect a close taxonomic relationship. However, this need not be so, provided that the cross reacting antigen is a good immunogen present in sufficient quantities and suitably located on the surface. This is exemplified by the cross reaction between *Rickettsiae* and certain strains of Proteus.

The cross agglutination between *Brucellae* and *Yersinia enterocolitica* type IX observed in the present work is marked, since the titres with the cross reacting antigen were at the same level as the homologous titres, and the absorption with the cross reacting antigen reduced the homologous titres. This was observed both with sera from patients and with sera prepared in rabbits. The cross absorptions suggested in most instances that the infective agent had been *Yersinia enterocolitica* type IX rather than any brucella, and in no instance did they provide evidence to the contrary.

Based on a series of biochemical tests, type IX closely resembles the other subtypes of *Yersinia enterocolitica* (*Niléhn et al.* 1968). It differs from *Brucellae* in that it grows better on various media as well as anaerobically, is motile at 22° C (but not at 37° C) and produces acid in many of the conventional sugar fermentation tests.

The clinical picture of the disease in the patients has been analysed (*Ahvonen & Sievers* 1968) and found to be compatible with that observed in infections caused by other types of *Y. enterocolitica* (*Winblad et al.* 1966, *Mollaret* 1966). However, due to the diversity of symptoms observed in human brucellosis (*Elberg* 1965) the clinical picture in some cases also fitted in with that of brucellosis.

It is apparent that problems involved in the serological diagnosis of brucellosis arise in areas where infections caused by *Y. enterocolitica* type IX do occur.

SUMMARY

Twenty four human sera containing brucella agglutinins corresponding to 100–1600 IU had agglutination titres of the same level or slightly

higher against *Yersinia enterocolitica* type IX. Very weak or no reactions were obtained with other yersinia antigens and with tularaemia antigen. Cross absorptions suggested that the infective agent had been *Yersinia enterocolitica* type IX rather than any *Brucella* in the majority of cases. Rabbit antisera against these bacteria gave titres of the same level with both antigens.

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in six others the absorption with *Brucella abortus* removed all detectable agglutinins against *Y. enterocolitica* type IX.

Table 3 illustrates the cross absorption of two rabbit immune sera. Noteworthy is the very marked titre reduction of *Brucella abortus* immune serum after the absorption with *Y. enterocolitica* type IX. Similar types of results were obtained with the other immune sera.

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Acta path microbiol scandinav 75 295 304 1969

Institute for Experimental Pathology Keldur Reykjavik Iceland.

VISNA AND MÆDI VIRUS ANTIGEN IN INFECTED CELL CULTURES STUDIED BY THE FLUORESCENT ANTIBODY TECHNIQUE

By

HALLDOR THORMAR

Received 27 vi 68

Electron microscope studies have indicated that visna and mædi viruses are formed by budding at the surface of their host cells (*Thormar* 1961 *Thormar* 1965) but did not reveal any specific structural changes either in the nuclei or in the cytoplasm Observations of visna virus infected cell cultures stained with acridine orange suggested an increase in the amount of single stranded RNA in the cytoplasm of infected cells (*Thormar* 1966) The cytoplasm fluoresced uniformly orange red without much particulate staining The orange red fluorescence was remarkably intense in the cytoplasmic processes of stellate cells and at the surface of degenerating cells No specific changes were observed in the cell nuclei

The present study was undertaken in an attempt to learn more about host cell virus interactions in cell cultures infected with visna and mædi viruses particularly with regard to intracellular formation of virus antigen during the growth cycle

MATERIALS AND METHODS

Cell cultures Serial cultures of sheep choroid plexus cells (*Sigurdsson Thormar & Palsson 1960*) were grown in medium 199 containing 20 per cent sheep serum and maintained in medium 199 with 2 per cent sheep serum Monolayer cultures grown on 11 × 22 mm coverslips in Leighton tubes were used in all experiments

Infection of cultures with virus Visna virus strain K796 and mædi virus strain M88 were used (*Thormar* 1965 *Thormar & Helgadóttir* 1965) Each coverslip culture was infected with virus at an input multiplicity of about 10 $TCID_{50}$ per cell and rotated for 3 to 4 hours in a roller drum at 37 C The cell layers were then washed 3 times with medium 199 and 1 5 ml of maintenance medium added to each Leighton tube The tubes were rotated again at 37 C until harvested for fixation and staining of the cell layer and for titration of virus in the fluid medium (*Thormar & Helgadóttir* 1965)

Antisera A number of sheep antisera against visna and mædi viruses were collected from sheep at intervals varying from 5 weeks to 49 months after intracerebral or intrapulmonary inoculation with infective virus Sera from the same animals collected before inoculation with virus and sera from normal uninfected sheep were

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Author s present address New York State Institute for Basic Research in Mental Retardation Forest Hill Road Staten Island N Y 10314 U S A

The able technical assistance of Miss Helga Helgadóttir is gratefully acknowledged

used as controls The neutralization titres of the sera were determined as previously described (*Thormar* 1963 *Thormar & Helgadottir* 1965) The complement fixation titres were determined by the method of *Gudnadóttir & Kristinsdóttir* (1967) Serum globulins were prepared by precipitation with half saturated ammonium sulphate and were conjugated with fluorescein isothiocyanate according to the method of *Marshall Eveland & Smith* (1958) Fluorescein labelled globulins were absorbed 3 times with sheep liver powder and once with a suspension of fresh choroid plexus cells in order to eliminate as much as possible of non specific fluorescent staining

Fixation and fluorescent staining of cell cultures Washed cell layers were fixed for 10 minutes in dry acetone and stained by the direct fluorescent antibody technique (*Coons et al* 1942 *Coons & Kaplan* 1950) at 37 C for 45 minutes After thorough washing with phosphate buffered saline and mounting of the coverslips in buffered glycerol the cells were examined in a Leitz Ortholux fluorescence microscope with a darkfield condenser using primary filter BG12 and a blue light absorbing barrier filter Kodak Ektachrome high speed film was used for colour photography

Specificity of staining The following criteria were used as proof of specificity of the fluorescent staining (*Coons & Kaplan* 1950 *Cherry Goldman & Carski* 1960) 1) Conjugated antisera stained cultures infected with visna and maedi viruses but not uninfected cultures or cultures infected with vaccinia virus herpes simplex virus or Newcastle disease virus 2) Cultures infected with visna or maedi viruses were not stained by conjugated normal sera 3) Staining of infected cells was inhibited by preincubation of the cell layers with unconjugated visna or maedi antisera but not by preincubation with unconjugated control sera Antisera against maedi virus blocked staining by fluorescein labelled visna antisera and vice versa

RESULTS

Localization of virus antigen in cells harvested at successive stages of infection A series of coverslip cultures was harvested at intervals after inoculation with virus The cell layers were fixed for immunofluorescent staining and samples were removed from the fluid medium for virus titration Uninoculated control cultures were harvested for fixation and staining at the same intervals as infected cultures

There was no specific staining in cultures harvested 6 hours after infection with visna virus A diffuse specific fluorescence was observed in the cytoplasm of a small number of cells in cultures harvested 20 hours post infection (p i) shortly before a rise in visna virus titre was detected in the medium During the following hours both the number of fluorescing cells and the intensity of fluorescence increased concurrently with the increase in virus titre in the cultures In some cells the cytoplasmic fluorescence was seemingly most intense around the nuclei possibly because the thickness of the cytoplasm was greatest in the perinuclear area At 3 days p i about half of the cells showed cytoplasmic fluorescence It was most intense in stellate cells with a cytopathic effect characteristic for visna virus In a few cells bright fluorescent staining was observed at the cell surface apparently in the form of floccules of various sizes (Fig 1) At 4 days p i the floccules had become more pronounced and in some cells the cell periphery showed up as a bright line of varying thickness being particularly distinct in cytoplasmic processes of stellate cells (Fig 2) At least some of the antigen was apparently localized outside the cell membrane since

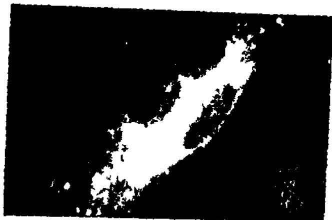


Fig 1
Three days after infection Diffuse cytoplasmic fluorescence and bright granules at the cell surface

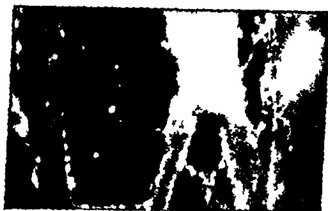


Fig 2
Four days after infection Bright fluorescence of the cell membrane



Fig 3
Fluorescent staining without fixation of cells harvested 4 days after infection Some virus antigen is localized on the outer surface of the cell membrane

Figs 1 to 4
Sheep cells stained with fluorescent antibodies at various times after infection with visna virus × 750



Fig 4
A cell rounding up 4 days after infection Intense fluorescence particularly of surface knobs



Fig 5
Cells stained 7 days after infection with mædi virus Cytoplasmic fluorescence particularly bright at the cell surface × 750

was stainable with fluorescent antibodies before fixation (Fig 3) Cells which were rounding up appeared very bright and their surface was covered with small knobs of brilliant fluorescence (Fig 4)

Specific fluorescence was not detectable in cultures infected with mædi virus until at least 30 to 40 hours p i and it increased slowly during the following days As in visna the fluorescence was confined to the cytoplasm and became particularly intense at the cell surface about 6 to 7 days p i (Fig 5) At no time was there evidence of specific nuclear fluorescence in cells infected with visna or mædi viruses

Fluorescent antibodies in various visna and mædi antisera Several visna and mædi antisera were tested for fluorescent antibodies and an attempt was made to compare the intensity of the fluorescence with the neutralization and complement fixation titres of the sera (Table 1) The fluorescent activities of various sera were compared in infected cell cultures under the same conditions and were scored as 2 + (bright fluorescence) + (distinct fluorescence) (+) (faint fluorescence) and — (no specific fluorescence) Preinoculation sera and other normal sera



Fig 1
Three days after infection Diffuse cytoplasmic fluorescence and bright granules at the cell surface



Fig 2
Four days after infection Bright fluorescence of the cell membrane



Fig 3
Fluorescent staining without fixation of cells harvested 4 days after infection Some virus antigen is localized on the outer surface of the cell membrane

Figs 1 to 4
Sheep cells stained with fluorescent antibodies at various times after infection with visna virus × 750

DISCUSSION

In the present work the localization of visna and mædi antigen in cells harvested at various times after inoculation was studied using a number of various fluorescein labelled antisera. A uniform specific fluorescense was observed in the cytoplasm of cells infected with visna and mædi viruses and later it increased to intensity at the cell surface indicating that virus antigen was formed in the cytoplasm and accumulated in high concentration at the cell membrane. The formation of detectable amounts of viral antigen in infected monolayers coincided with the formation of infectious virus in the cultures. Specific fluorescence could not be detected with certainty in the cell nuclei at any time during the viral growth cycle regardless of the antiserum used for fluorescent staining. Either there was no viral antigen synthesized in the nuclei of infected cells or such an antigen failed to cause detectable antibody formation in sheep.

The results of the present study are in general agreement with a recently published report by *Harter, Hsu & Rose* (1967), except that large cytoplasmic inclusions of fluorescent material were not observed at any time during the infection and viral antigen seemed to aggregate only at or outside the cell membrane.

A comparison of the information obtained by electron microscopy and by immunofluorescent and acridine orange staining of cells infected with visna virus indicates that the cytoplasm is the main site of formation of virus material in host cells. Electron microscope observations have shown that in a late stage of visna and mædi infections when most of the cells in a monolayer have either been turned into stellate forms or are rounding up and detaching from the glass, virus like particles are found in a large number on the surface of the cells and the cell membranes are covered with small buds which are believed to be virus particles being formed and released by the cells (*Thormar* 1961, *Thormar* 1965). At this stage virus antigen is present in large amounts at the cell surface and partly outside the cell membrane, as shown by immunofluorescent staining of unfixed cell surfaces. At the same time cytoplasmic processes of stellate cells and the surface of degenerating cells stain brilliantly orange red by acridine orange (*Thormar* 1966), indicating high concentration of single stranded RNA. All these observations indicate that large amounts of viral antigen and RNA are being incorporated into virus particles at the cell membrane. However, the conclusion that visna and mædi virus particles contain RNA is yet to be confirmed by biochemical methods.

Sheep infected with visna and mædi viruses seem to form antibodies detectable by fluorescein labelling shortly after infection and long before neutralizing antibodies are detectable in their sera. It is not known whether the fluorescent antibodies are identical to the complement fix

TABLE 1

A Comparison of Fluorescent Antibody (FA) Activity and Neutralization (N) and Complement Fixation (CF) Titres of Various Visna and Mædi Antisera and Normal Sheep Sera. The FA Activity was Estimated from the Intensity of Specific Fluorescence in Visna and Mædi Virus Infected Cell Cultures stained with Fluorescein-labelled Serum Globulins

Serum	Time of collection	N titre against		CF titre against		FA activity against	
		visna virus	mædi virus	visna virus	mædi virus	visna virus	mædi virus
Pre sera	Before inoculation	<4	<4	<4	<4	–	–
Anti visna No 5870	5 weeks p i*	<4	<4	8	8	(+)	(+)
Anti visna No 6040	4 months p i	<4	<4	128	32	2+	+
Anti visna No 6873	12 months p i	256	64	128	32	2+	+
Anti visna No 8752	49 months p i	1024	256	256	64	2+	+
Anti visna No 4992	21 months p i	1024	<4	128	32	2+	(+)
Anti mædi No 14663	30 months p i	256	256	128	128	2+	2+
Normal sera		<4	<4	<4	<4	–	–

* Post inoculation

were consistently found to be free of specific fluorescent antibodies against visna and mædi viruses as well as of neutralizing and complement fixing antibodies. A very slight fluorescent activity was found in a serum which was collected 5 weeks after intrapulmonary (i.p.) inoculation with a high tissue culture passage of visna virus (serum no 5870). This serum had a very low complement fixing activity and no neutralizing activity against the virus. All the other post inoculation sera were consistently found to contain fluorescent antibodies against the viruses. These sera were collected from sheep after i.p. inoculation with a high tissue culture passage of visna virus (sera no 6040 and no 6873), after intracerebral (i.c.) inoculation with a low tissue culture passage of visna virus (serum no 8752), after i.c. inoculation with a low tissue culture passage of mædi virus (serum no 14663) and after i.c. inoculation with extract from a visna brain (serum no 4992). The intensity of the fluorescent staining was found to correlate fairly well with the complement fixation titres of the sera. There was, on the other hand, no correlation between fluorescent staining and the neutralization titres of the sera. Fluorescein labelled mædi antisera stained the cytoplasm of visna virus infected cells and vice versa. However, visna antisera seemed to have somewhat less fluorescent activity against mædi virus than against visna virus.

DISCUSSION

In the present work the localization of visna and mædi antigen in cells harvested at various times after inoculation was studied using a number of various fluorescein labelled antisera. A uniform specific fluorescense was observed in the cytoplasm of cells infected with visna and mædi viruses and later it increased to intensity at the cell surface indicating that virus antigen was formed in the cytoplasm and accumulated in high concentration at the cell membrane. The formation of detectable amounts of viral antigen in infected monolayers coincided with the formation of infectious virus in the cultures. Specific fluorescence could not be detected with certainty in the cell nuclei at any time during the viral growth cycle regardless of the antiserum used for fluorescent staining. Either there was no viral antigen synthesized in the nuclei of infected cells or such an antigen failed to cause detectable antibody formation in sheep.

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ing antibodies which are also formed shortly after infection with visna and maedi viruses

SUMMARY

The development of virus antigen in sheep cell cultures infected with visna and maedi viruses was studied by the fluorescent antibody technique A diffuse specific fluorescence was observed in the cytoplasm of infected cells shortly before the formation of new infectious virus Later the cytoplasmic fluorescence increased in intensity and became particularly brilliant at the cell surface indicating that virus antigen was formed in the cytoplasm and accumulated in high concentration at the cell membrane coinciding with maximum virus titre in the cultures At no time was there evidence of specific nuclear fluorescence in infected cells

In a number of sheep sera fluorescent activity was found to correlate with the complement fixation titre but not with the neutralization titre Fluorescein labelled maedi antisera stained cells infected with visna virus and vice versa

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Acta path microbiol scandinav 75 303-31? 1969

The Institute of General Pathology and the Department of Neurology Aarhus Kommunehospital University of Aarhus Denmark

THE OCCURRENCE OF VIRUS AND INTERFERON IN SPLEEN, SERUM AND BRAIN IN STEROID TREATED MICE UNDER EXPERIMENTAL INFECTION WITH WEST NILE VIRUS

By

SVEN HAAHR

Received 13 vii 68

The effect of steroids on interferon production in cells infected with virus has been studied several times *Kilbourne et al* (10) demonstrated in 1961 that cortisone inoculated into the allantoic cavity prior to infection with influenza virus inhibited the production of interferon and in 1963 *deMaeyer & deMaeyer* (12) reported that steroids had a similar effect on interferon production in tissue culture Later studies have given corresponding results (17 18)

In animal studies *Mendelson & Glasgow* (13) and *Rytel & Kilbourne* (20) have shown that the amount of circulating interferon is reduced in steroid treated mice compared with control mice after the injection of a large number of virus particles In addition there was less interferon in the spleen and lung of the cortisone treated mice (20) In one of the studies interferon induction was accomplished with Newcastle disease virus which does not multiply in the murine organism following intravenous inoculation (20) while in the other study (13) interferon was only measured during the first 6 hours after inoculation with Sindbis virus that is during a period where interferon is apparently only slightly influenced by virus multiplication in the organism s cells

In the work presented here the effect of steroid treatment on mice infected with West Nile virus a neurotropic arbovirus was studied The virus and interferon content of the serum spleen and brain was investigated throughout the cour[illegible] of the infection Sensitivity of the mice to the virus during ster[illegible] t[illegible] nt was also studied as well as the effect of steroid tre[illegible]tment [illegible] white blood cell picture and the weight of the spleen

MATERIA[illegible] METHODS

[illegible] and Semliki forest disease virus strain [illegible] Casals in 1961 A description of passages [illegible] a previous paper ( ) Mice were injected

intraperitoneally or intracerebrally with about 10^3 LD_{50} (as determined by intra cerebral inoculation in suckling mice) of West Nile virus in volumes of 0 05 ml suspended in phosphate buffered saline (PBS)

Tissue culture Mouse embryonic cells were prepared from embryos 18-20 days old from albino mice as previously described (8) The cells were grown in Eagle s Basal Medium (*Gibco*) with 10 per cent inactivated calf serum and antibiotics in 1 litre Roux flasks

Animals Male and female albino mice of a non inbred strain weighing 16-19 g were used in all the experiments Ten mice were used in each experimental group

Processes of specimens Removal of blood spleen and brain was done as de scribed previously (8) The pooled blood was centrifuged at 3000 r p m for 10 mi nutes The serum was pipetted off and used for virus titration and for interferon assay The pooled organs were stored at — 70 C A 10 per cent suspension was made from the frozen organs by grinding with sand in PBS After centrifugation at 6000 r p m for 30 minutes the supernatant was withdrawn and used for virus titration and interferon assay

Steroid Hydrocortisate (Hydrocortisoni acetas NLN Lovens kemiske Fabrik Copenhagen) suspended in water was administered subcutaneously near the tail root in doses of 1 mg or 10 mg per mouse 24 hours prior to inoculation with West Nile virus Control mice were inoculated the same way with equivalent doses of PBS

Virus titration The virus content of the serum and organs was determined by intra cerebral inoculation into mice aged 2 to 4 days in 10 fold serial dilutions in PBS The virus titre was expressed as the exponent of the logarithmic ($\log_{10}$) dilution per 10 mg of organ or serum which caused death in 50 per cent of the animals as calculated by the method of *Kärber* (11) The sensitivity of the experimental ani mals to the neurotropic West Nile virus on intraperitoneal and intracerebral ino culation was studied by inoculation of the virus in 4 fold serial dilutions in PBS. The virus titre was expressed as the exponent of the logarithmic ($\log_{10}$) *dilution* per 0 05 ml of virus which caused death in 50 per cent of the animals

Interferon assay Serum dilution and organ extracts were dialysed against Soren sen s buffer pH 2 0 and after 48 hours at 4 C dialysed back to pH 7 4 After centri fugation at 3000 r p m for 30 minutes the supernatant was used for the assay Determination of anti viral activity was performed by the plaque inhibition method in secondary cultures of mouse embryonic cells as described previously (8) Inter feron titres expressed as units per 200 mg of serum or organ were recorded as the reciprocal of the highest dilution which reduced by 50 per cent the number of plaques counted in the controls i e 50 per cent plaque depressing dose (PDD_{50}/ 200 mg)

A stock reference preparation of interferon at known titre was used on each assay to detect any changes in the sensitivity of the system

Characteristics of viral inhibitor The viral inhibitor found in the serum dilutions and organ extracts exhibited the characteristics described in a previous paper (8) *The properties are in keeping* with mouse interferon as described by other in vestigators (6)

Histology Spleens were fixed in 10 per cent formalin and 4 micron *sections were* cut and stained with haematoxylin and eosin

Leucocytes and differential counts Venous blood was obtained by incision *in the* tail Blood used for leucocyte counts was diluted 1 20 in methyl violet acetic acid and counting was done in a Burger Turk chambre Differential counts were per formed on blood smears stained with May Grunwald Giemsa stain

Serum hydrocortisone assay Was carried out by Medicinsk Laboratorium *Copen* hagen using a double isotope derivation technique *with steroids and coupling with* S S pipsan

RESULTS

The virus and interferon content of the serum spleen and brain was studied during the entire course of infection after intraperitoneal in jection of West Nile virus *Figs 1 2 and 3* give the content of *interferon* and virus in serum spleen and brain respectively in *mice treated* with 10 mg of hydrocortisate or a corresponding volume of *salt solution*

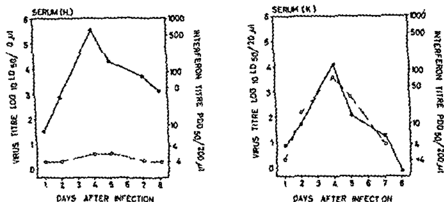


Fig 1

Titres of virus (●——●) and interferon (○- - -○) in serum of mice 16–19 g treated with 10 mg of hydrocortisate (H) or a corresponding volume of salt solution (K) 24 hours before intraperitoneal injection of West Nile virus

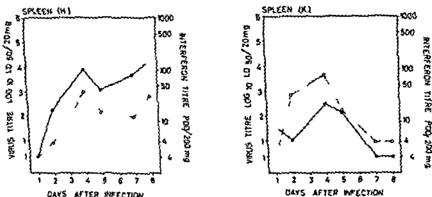


Fig 2

Titres of virus (●——●) and interferon (○- - -○) in the spleen of mice 16–19 g treated with 10 mg of hydrocortisate (H) or a corresponding volume of salt solution (K) 24 hours before intraperitoneal injection of West Nile virus

Only a trace of virus inhibiting activity was found on the 4th and 5th day in the serum of steroid treated mice in the interferon assay studies. This was in contrast to the findings in control mice where a marked increase in the serum concentration of interferon was seen from the second day with a maximum titre of 80 on the fourth day.

In spleens from the steroid treated mice no virus inhibiting activity was found the first two days but thereafter there was a rising concentration. From the fourth day the values were of the same order of magnitude per weight unit as in the controls. In these animals the interferon content was relatively high already two days after virus injection, the titre subsequently rising to a maximum on the fourth day, followed by a more pronounced fall than that [illegible] the steroid animals.

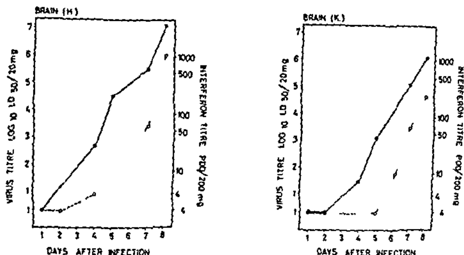


Fig 3

Titres of virus (●——●) and interferon (○- - -○) in the brain of mice 16–19 g treated with 10 mg of hydrocortisate (H) or a corresponding volume of salt solution (K) 24 hours before intraperitoneal injection of West Nile virus

In the brain the changes in interferon content were similar in the two groups but interferon could be demonstrated somewhat earlier in the steroid treated mice

The virus content of the serum was found to be higher in steroid treated mice than in controls viraemia rose more rapidly in the former and remained at a higher level for a longer time Essentially the same was the case as concerns the spleen In the brain a sharply rising virus titre with a maximum value which was found to be significantly higher in the steroid treated mice than in the controls

The results obtained in mice treated with 1 mg of hydrocortisate prior to virus injection were essentially the same as those found in mice pretreated with 10 mg of hydrocortisate but the virus and interferon

TABLE 1

Virus and Interferon Content in the Brain and Serum after Intracerebral Inoculation of West Nile virus in Mice Treated with 10 mg of Hydrocortisate and in Untreated Mice

Days after inoculation of virus	Brain				Serum			
	Virus		Interferon		Virus		Interferon	
	H	C	H	C	H	C	H	C
1	3 5§	3 7	100†	100	? 3	3 0	4	64
2	4 7	4 5	1600	1600	3 5	3 3	9	40
3	6 1	4 5	3200	3200	4 1	2 9	8	40
4	6 3	6 5	3?00	3200	4 5	3 1	4	64

H = hydrocortisate C = untreated mice
§ Virus = $\log_{10}$/20 mg
† Interferon = PDD_{50}/200 mg

content in the brain at the end of the course of infection presented no clear difference between the two groups

The virus and interferon content in the brain after intracerebral injection of West Nile virus in mice treated with 10 mg of hydrocortisate is given in Table 1 As indicated the results in the two groups were practically identical A persistently reduced interferon content was found in the serum and after the first day an increased virus content

TABLE 2

Hydroco ticate Concentration in the Serum of Mice after Treatment with 1 mg and 10 mg of Hydrocortisate

Hours after treatment with hydrocortisate	1 mg of hydrocortisate	10 mg of hydrocortisate
24	0 28 microgram/ml	1 2[illegible] microgram/ml
72	0 16 microgram/ml	0 50 microgram/ml
120	0 0[illegible] microgram/ml	0 57 microgram/ml
168	0 04 microgram/ml	0 34 microgram/ml
Untreated mice	0 01 microgram/ml	

The hydrocortisate concentration in the serum after treatment with 1 and 10 mg of hydrocortisate is given in Table 2 Measurement was done on non infected mice and each measurement was performed on pooled serum from 10 mice A considerable elevation in hydrocortisate concentration was found 24 hours after hydrocortisate injection upon which values decreased After treatment with 1 mg of hydrocortisate a clearly elevated serum concentration was found during the first 3 days normal values being slightly elevated during the following days After treatment with 10 mg of hydrocortisate serum values were clearly elevated during the entire period of infection as normally produced by West Nile virus

The effect of steroid treatment on sensitivity to the virus was found to be dependent upon the route of virus inoculation As shown in

TABLE 3

Sensitivity to West Nile Virus Measured as log LD, after Intraperitoneal and Intracerebral Inoculation in Hydrocortisate Treated and Untreated Mice on Two Different Titrations

Route of virus inoculation	hydrocortisate treatment	Titration 1	Titration 2
Intraperitoneal	10 mg hydroc	5 8 ± 0 [illegible]	
Intraperitoneal	1 mg hydroc	5 1 ± 0 [illegible]	5 4 ± 0 2
Intraperitoneal	no treatment	4 5 ± 0 [illegible]	
Intracerebral	10 mg hydroc	5 9 ± 0 2	4 [illegible] ± 0 2
Intracerebral	no treatment	5 6 ± 0 4	5 8 ± 0 [illegible]
Suckling mice intracerebrally without hydrocortisate treatment		6 1 ± 0 2	5 7 ± 0 [illegible] 6 0 ± 0 [illegible]

Table 3 sensitivity to virus after treatment with 10 mg of hydrocortisate was markedly increased after intraperitoneal inoculation but only slightly increased or unchanged after intracerebral injection. It is also shown in Table 3 that treatment with 1 mg of hydrocortisate increased sensitivity to virus after intraperitoneal virus injection.

Hydrocortisate treatment by itself caused growth inhibition and in some mice weight loss during the first days after it was given but otherwise it did not affect the mice's general condition. The only deaths seen among the steroid treated mice occurred among those that had been inoculated with virus.

TABLE 4

Leucocyte and Differential Counts Done on Mice Treated with 1 mg of Hydrocortisate (H) and Untreated Mice (C). Average of Counts in 6 Mice

Hours after treatment with hydrocortisate	Leucocyte counts		Differential counts			
			% polymorphs		% lymphocytes	
	H	C	H	C	H	C
24	2093	5840	34	15	66	8[illegible]
48	19[illegible]0	3197	41	13	59	8[illegible]
96	2[illegible]38	3391	74	2[illegible]	[illegible]	78
120	[illegible]900	3900	79	19	[illegible]1	81
168	41[illegible]8	45[illegible]4	80	1[illegible]	20	84
192	4[illegible]0	3445	79	[illegible]3	[illegible]1	7[illegible]

TABLE 5

Weight of Spleen in Virus infected Mice Treated with 10 mg of Hydrocortisate (H) and Untreated Mice (C). Average Weights of Spleens from 10 Mice

Hours after treatment with hydrocortisate	H	C
48	32 mg	110 mg
72	30 mg	100 mg
1[illegible]0	3[illegible] mg	163 mg
144	30 mg	170 mg
192	37 mg	161 mg

Leucocyte and differential counts in mice after administration of 1 mg of hydrocortisate are given in Table 4. The first counts were carried out 24 hours after steroid injection. There was a slight fall in leucocyte count with lymphopenia during the first days following steroid injection. A similar phenomenon was seen in mice treated with 10 mg of hydrocortisate, the changes seen however being somewhat more pronounced. There was a greater fall in leucocyte count and fully developed lymphopenia 24 hours after steroid administration.

The weight of the spleen in untreated mice and in mice treated with 10 mg of hydrocortisate is given in Table 5. A marked reduction in the

weight of the spleen was seen in the steroid treated mice A similar weight reduction was seen in mice treated with 1 mg of hydrocortisone On histological examination of spleens from steroid treated mice a substantial reduction in lymphoid tissues was seen

DISCUSSION

A reduction in circulating interferon in steroid treated mice has previously been demonstrated in the first hours after the intravenous injection of large amounts of virus (13 20) and in one of these studies the interferon content of the spleen and lungs was reduced (20)

In the work reported here a persistent reduction in the circulating interferon during the entire course of infection was found in steroid treated mice after intraperitoneal injection of West Nile virus

The reason for this could be the inhibitory effect of steroids on interferon formation in cells as has been demonstrated in *in vitro* and *in ovo* studies (10 12 17 18) However the pronounced interferon formation in brain cells in the steroid treated mice which was particularly marked when virus was injected intracerebrally speaks against this theory In addition the interferon content per weight unit in the spleens of steroid treated mice on the fourth day was nearly as great as the interferon content in the control mice This apparently means that interferon production in intact spleen cells at this point of time was only slightly reduced Indeed only a slight reduction in interferon formation was demonstrated in different organ systems at times when the amount of circulating interferon was markedly reduced

Thus it seems reasonable to conclude that steroid treatment has a specific destructive effect on tissues contributing in a major way to the circulating interferon A cytolytic effect on lymphoid tissue is known from previous studies (5) and in the present study a reduction in circulating lymphocytes and a substantial reduction in the weight of the spleen which seemed to result primarily from a disappearance of lympoid tissue was found Presumably this circumstance can be taken as evidence of a generalized effect on lymphoid tissue throughout the organism

The reticuloendothelial system and/or the lymphoid tissue have previously been considered to contribute in a high degree to the circulating interferon (8 19) This theory is supported by the present investigation

Steroid treatment has essentially the same effect on lympoid tissue in all animals (5) and the adverse effect of steroid treatment on virus ressistance in man (3 4 15) can perhaps be a consequence of this action

Whether the increased viraemia in the steroid treated mice is solely a result of the reduction in circulating interferon is impossible to determine *In vitro* and *in ovo* studies almost always show an unchanged production of infectious virus when interferon formation is reduced

by steroid treatment (13 17 18) but direct comparison is hardly permissible

In animal studies increased virus content in organs (9 21) has been encountered in several instances as a result of steroid treatment interferon measurement was not however undertaken in these studies In animal studies in which a reduced quantity of circulating interferon has been demonstrated (13) no studies of virus content were performed In mice inoculated with Sindbis virus where the amount of circulating interferon was reduced by treatment with Actinomycin D increased viraemia was found (16)

The increased content of virus in the spleen is difficult to explain Increased phagocytosis of virus in the spleens of steroid treated mice has been reported by one investigator (1) but most workers find that phagocytosis is reduced after steroid treatment (14 22)

In this study the sensitivity to virus was found to be clearly increased in steroid treated mice after intraperitoneal injection of virus This was not or only slightly the case after intracerebral virus inoculation An exhaustive explanation of this can scarcely be given Clearly several factors are important but in intraperitoneally infected mice treated with steroids which results in a reduction in the amount of circulating interferon and increased viraemia the virus has presumably a greater tendency to invade the central nervous system giving a more intense infection of the brain On the other hand using intracerebral inoculation by which virus is applied directly to the target organ the infection will probably be influenced only to a minor degree by the increased viraemia and the reduction in the amount of circulating interferon resulting from steroid treatment

The results obtained in this study agree with these suppositions and suggest that the effect of steroids on the course of infection with neurotropic virus is exerted at organic sites other than the target organ

The importance of antibody production for the course of a primary virus infection is as yet an unsolved problem (2) Most investigators however consider antibody production to be of no or only little importance and antibody measurements were not performed in the present study

SUMMARY

In mice treated with 10 mg of hydrocortisone subcutaneously 24 hours before intraperitoneal injection of West Nile virus the content of interferon in serum was found to be markedly reduced during the entire course of infection (8 days) together with a reduced interferon concentration in the spleen during the first two days after virus injection but with no change in interferon production in the brain Increased amounts of virus was found in the serum spleen and brain and an increased sensitivity to virus was demonstrated In mice treated with 10 mg of hydrocortisone subcutaneously 24 hours before intracerebral virus in

jection no definite changes in virus and interferon content were found in the brain but a reduced interferon content and an increased virus content were demonstrated in the serum Sensitivity to virus was unchanged or only slightly increased

Steroid treatment produced lymphopenia and a reduction in weight of the spleen

These studies seem to support the theory that the reticulo endothelial system and/or the lymphoid tissue contribute appreciably to the circulating interferon In addition this investigation suggests that the effect of steroids on the course of infection with neurotropic virus is exerted in the organism outside the target organ

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Acta path microbiol scandinav 75 313 3?? 1969

Statens Seruminstitut Regional Laboratory Odense City and County Hospital
(Head K F Sib ni M D) Str Boulevard 29 Odense Denmark

FLAVOBACTERIUM MENINGOSEPTICUM ISOLATED FROM OUTSIDE HOSPITAL SURROUNDINGS AND DURING ROUTINE EXAMINATION OF PATIENT SPECIMENS

By

HELGE OLSEN

Received 2? viii 68

The present study consists of an examination of the incidence of *Flavobacterium meningosepticum* in tap water brook water and cultivated soil The specimens originated from the town of Odense and its immediate neighbourhood Furthermore the frequency in specimens from patients without clinical signs of infection with *Flavobacterium meningosepticum* was investigated

In a few cases *Flavobacterium meningosepticum* had caused meningitis in premature infants (surveys see *Olsen* 1967 c) and bacteraemia in adults (*Olsen* 1967 a) The microorganism was first classified as a species by *King* (1959) Strains resembling *Flavobacterium meningosepticum* but showing deviation as regards their acid production from carbohydrates have since been described (*King* 196?)

METHODS

Biochemical Properties

Flavobacterium meningosepticum is a gram negative non motile rod The biochemical properties have been described previously (*King* 1959 *Olsen et al* 1965 *Olsen* 196?) Mention will only be made here of the properties required in the present study in order to [illegible] a strain identical with *Flavobacterium meningosepticum* The [illegible] the same as the properties of the strain that caused bacteraemia in [illegible] (*Olsen* 196? a)

1 Th [illegible] h [illegible] b a [illegible] an [illegible] n motile
Th [illegible] (Ko a s test) produce indol, liquefy gelatin an [illegible] reduce [illegible]
1 [illegible] h [illegible] tta ked [illegible] x [illegible] Acid production from [illegible] carbo-
h [illegible]

Requests for reprints should be addressed to Dr Helge Olsen Diagnostic Bacteriolog Department Statens Seruminstitut Amager Boulevard 80 2300 København S Denmark

Acid produced	Acid not produced
glucose	xylose
lævulose	arabinose
maltose	rhamnose
lactose	galactose
trehalose	sucrose
mannitol	raffinose
glycerol	starch
ethanol	adonitol
	dulcitol
	sorbitol
	inositol
	inulin
	salicin

Indol production was examined after incubation for two days at 35 C The indol was first extracted by shaking with xylene then overlayed with Ehrlich Bohme s reagent (*King* 1959)

Hugh & Leifson s medium (1953) was used for the investigation of acid production from carbohydrates The tubes were observed for 30 da s at 35 C

Sensitivity to Antibiotics

The antibiotic sensitivity pattern was peculiar and corresponded somewhat to that of a gram positive bacterium (*Olsen* 1967 c) Only strains with this characteristic sensitivity pattern were considered to be identical with *Flavobacterium meningosepticum*

In the present investigation the sensitivity to eight antibiotics was determined using an agar diffusion method with tablets containing antibiotics (Sensitabs Rosco®) Using this method 13 strains of *Flavobacterium meningosepticum* showed the following sensitivity to antibiotics (the figures in brackets indicate the content of antibiotic in the tablets)

sulphamethizole (10 mg)	0	
penicillin (15 µg)	0	
polymyxin B (5 mg)	0	
tetracycline (1 mg)	+	
chloramphenicol (500 µg)	+	++
streptomycin (1 mg)	+	++
kanamycin (1 mg)	+	++
erythromycin (500 µg)	++	+++

The considerable resistance to antibiotics of *Flavobacterium meningosepticum* was utilized for the isolation However the patient specimens were is lated without the use of antibiotics Water specimens were filtered through a membrane filter which was subsequently placed on the medium (Sartorius membrane filter 11406–50 mm Germany) Several media and methods were examined befor the following method was selected as the most suitable

1 The specimens was inoculated on to 5 per cent horse blood agar containing 10 I U of penicillin + 40 µg of streptomycin + 40 µg of polymyxin B per ml Incubation for two days at 35 C All colonies ith different morpholc g were studied further

2 Pure cultivation using *Conradi Drigalski* plates Incubation for two days at 35 C

3 Inoculation into semisolid agar and examination for acid production from mannitol and salicin at 35 C (Hugh & Leifson medium)

4 Aerobic non motile oxidase positi e strains which attacked mannitol but not alicin within four days were studied with a view to indol production gelatin liquefaction and nitrate reduction

The strains that produced indol liquefied gelatin and did not reduce nitrate had carbohydrate and antibiotic sensitivity patterns similar to tho e of *Flavobacterium meningosepticum*

RESULTS

Using the method described 72 strains among which 14 were different were isolated from 32 soil specimens These strains were aerobic non motile and oxidase positive produced indol liquefied gelatin and did not reduce nitrate Seventy strains had the characteristic antibiotic sensitivity pattern of *Flavobacterium meningosepticum* No strain was completely identical with *Flavobacterium meningosepticum* as regards acid production from carbohydrates since invariably acid production from lactose was lacking Other deviations were quite small however and consisted mainly of acid production from starch sucrose and ethanol (Table 2)

Using other less selective methods 122 different aerobic non motile oxidase positive strains were isolated from the four sources mentioned The majority of these differed from *Flavobacterium meningosepticum* in several biochemical respects and in their sensitivity to antibiotics but as shown in Table 1 five strains were isolated that were identical with *Flavobacterium meningosepticum* Furthermore 13 strains differed only in not being able to produce acid from lactose and ethanol However this may be regarded as a minor deviation since most *Flavobacterium meningosepticum* strains attack lactose late and one strain did not attack ethanol at all (*Olsen* 1966)

TABLE 1

Flavobacterium meningosepticum *and other Aerobic Oxidase Positive Non Motile Strains Isolated from Four Different Sources*

Source	Total no of strains	No of different strains	Properties as *Fl mening*	Differing from *Fl mening* in not attacking: Lactose	Ethanol	Lactose and ethanol
Soil (79 specimens examined)	78	50	1		6	1
Brook water (93 ml examined)	60	46	3	0		1
Patient specimens (26 examined)	6	21	1	3		0
Tap water 40 litres examined)	5	5	0	0	0	0
Total	169	122	5	5	6	2

Flavobacterium meningosepticum was detected in brook water soil (from a garden) and patient specimens but not in tap water

The patient specimens were from blood urine sputum and spinal fluid In none of the cases could the clinical state of the patient be attributed to the presence of *Flavobacterium meningosepticum* The strains were therefore probably contaminants or saprophytes The

strain completely identical with *Flavobacterium meningosepticum* was from sputum from a patient in a Copenhagen hospital. The remaining patient specimens were from the Odense County and City Hospital. Six strains were isolated from urine, five from sputum, five from blood and two from spinal fluid.

Table 2 shows the carbohydrate reactions of all the indol positive, gelatin liquefying, non nitrate reducing strains. Groups containing strains isolated by the method described are marked with an [illegible]. The reactions with rhamnose, adonitol, dulcitol, sorbitol, inositol, inulin and salicin are not stated, since these seven reactions were always negative. On the basis of their carbohydrate reactions the 137 strains were distributed into the 44 groups stated.

TABLE 2

Carbohydrate Reactions of 137 Aerobic, Oxidase Positive, Non Motile Strains which in Addition were Indolpositive, Gelatin Liquefying, not Nitrate Reducing

No. of strains	Attacked by *Fl. mening.*								Not attacked by *Fl. mening.*						
	Glucose	Levulose	Maltose	Lactose	Trehalose	Mannitol	Glycerol	Ethanol	Arabinose	Xylose	Galactose	Sucrose	Starch	Raffinose	Yellow pigment
5	+	+	+	+	+	+	+	+	—	—	—	—		—	+
18	+	+	+	—	+	+	+	+	—	—	—	—	—	—	+
6	+	+	+	+	+	+	+	—	—	—	—			—	+
1	+	+	+	+	+	—	+	+	—	—		—		—	+
1	+	+	+	+	+	(+)	+	—	—			—		—	+
1	+	+	+		+	(+)	+	+	—	—		—		—	+
5	+	+	+	—	+	+	+			—	—			—	+
1	+	+	+		+	(+)	+	—	—	—		—			+
1	+	(+)	+		+	+	+		—				(+)		+
5	+	(+)	+	—	+	(+)	+	—	—		—		—	—	+
7	+	+	+		+	+	+	+	—	—			+		+
6	+	+	+		+	+	+	+				+		—	+
17	+	+	+	—	+	+	+	+	—	—			+	—	+
1	+	+	+		+	+	—	+	—						+
1	+	+	+		+	+	+	—		—		—	(+)		+
10	+	+	+	—	+	+	+	—		—	—		+	—	+
3	+	+	+	—	+	+	+	—	—	—		+		—	+
1	+	+	+		+	+	+	+	(+)	—			—	—	+
12	+	+	+	—	+	+	+	—	—	—	—	[illegible]	+		+
1	+	+	+		+	—	+	+	—	—		—	(+)		+
1	+	(+)	+		+	—	+	+	—	—	—	+	—	—	+
1	+	(+)	+		+	(+)	+	+	(+)	—		—		—	+
1	+	+	+		+	+	+	+	(+)	(+)	—	+	+	—	+
1	+	+	+		+	+	+	+	+	+		+		—	+
2	+	+	+		+	+	+	—		—	—		+	—	+
1	+	+	+		+		+	+	+	+				—	+
[illegible]	+	—	+	—	+	—	+	—		—	—		—	—	+
1	+	—	+	—	+	+	+		—	—	—		+	—	+
1	+	—	—	—	+	+	—		—		—		—	—	+
1	+	—	+	—	—	—	+	+		—		—	—	+	+
1	+	+	+	—	+	+	+	—	—	—	+	+	—	—	—
1	+	+	+	—	+	+	+			+		+	+		

TABLE (cont.)

No. of strains	Attacked by Fl. mening.								Not attacked by Fl. mening.							
	Glucose	Laevulose	Maltose	Lactose	Trehalose	Mannitol	Glycerol	Ethanol	Arabinose	Xylose	Galactose	Sucrose	Starch	Raffinose	Yellow pigment	
1	+	—	+	—	+	—	+	+	—	—	—	—	—	—	+	
1	+	+	+	—	+	—	+	—	—	—	—	+	+	—	+	
5	+	—	+	—	+	—	+	—	—	—	—	—	+	—	+	
3	+	—	+	—	—	—	+	+	—	—	—	—	+	—	+	—
[illegible]	+	—	+	—	—	—	+	—	—	—	—	—	+	—	+	
1	+	+	+	—	+	—	+	—	+	—	—	+	+	—	+	
1	+	—	+	—	+	—	+	+	—	—	—	+	+	—	+	
1	+	—	+	—	+	—	+	—	—	—	—	+	+	—	+	
2	+	—	+	—	—	—	—	—	—	—	—	—	—	—	—	+
1	+	—	+	—	—	—	—	—	—	—	—	—	+	—	—	
[illegible]	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	
1	+	+	+	(+)	+	—	—	+	—	+	+	+	—	—	+	
Totals 44 groups 137 strains																
Pos. reactions	43	27	43	4	38	21	39	20	3	4	2	15	16	1		
Weak reactions	0	4	0	1	0	5	0	0	3	1	0	0	3	0		
Neg. reactions	1	13	1	39	6	18	5	24	38	39	42	29	25	43		

On the basis of the carbohydrate reactions the strains disperse in the 44 groups stated in the table.

The reactions were also studied with rhamnose, adonitol, dulcitol, sorbitol, inositol, inulin and salicin. These seven reactions were for all the strains negative.

Yellow pigment was produced by the majority of the strains.

(+) means weak reaction.

Groups containing strains isolated by means of the selective method described in the text.

The most frequent deviations from *Flavobacterium meningosepticum* were due to acid production from starch and sucrose and no production or only weak acid production from lactose, ethanol, mannitol and laevulose.

The majority of the strains produced a yellow, non diffusible pigment. Most of the strains that did not produce pigment were from patient specimens.

Thirteen indol producing strains were found that deviated from *Flavobacterium meningosepticum* as regards gelatin liquefaction and nitrate reduction. Most of these were more active against carbohydrates than *Flavobacterium meningosepticum*, since they attacked galactose, raffinose, adonitol or salicin.

Sixty five different indol negative [illegible] ains were isolated which show still more deviating carbohydr[illegible] terns.

Statistical analysis of 117 [illegible] obic non motile oxidase posi[illegible]

The analysis was carried out at the [illegible] tatistical Department [illegible] institut

TABLE 3

117 Aerobic Non Motile Oxidase Positive Strains out of which 38 Indol Positive Gelatin Liquefying not Nitrate Reducing Strains were Compared with the 79 Remaining ones in Order to Learn whether a Difference was Present in Respects of Attacking the Same Carbohydrates as Flavobacterium meningosepticum

Method of isolation	Number of agreements with the carbohydrates pattern of 11 mening	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Total no of strains
I	Indolproducing gelatin liquefying not nitrate reducing strains	0	0	0	0	0	0	1	2	3	1	1	0	2	0	10
	Remaining strains	0	0	0	0	4	1	5	0	0	0	0	0	0	0	10
II	Indolproducing gelatin liquefying not nitrate reducing strains	0	0	0	0	0	0	3	1	3	1	2	0	1	0	11
	Remaining strains	1	0	6	3	3	14	7	3	0	1	0	0	0	0	38
III	Indolproducing gelatin liquefying not nitrate reducing strains	0	0	0	0	0	0	0	1	1	8	3	4	0	0	17
	Remaining strains	0	2	8	6	1	3	2	1	1	3	2	1	1	0	31
															Total	117

Twentyone carbohydrates were investigated The strains were divided into the groups I II and III on the basis of the antibiotics used for the isolation Groups I was isolated without the use of antibiotics (strains from patient specimens) group II was isolated by penicillin + polymyxin and group III by penicillin + streptomycin + polymyxin Each of the three groups was divided into two subgroups one comprising the indol positive gelatin liquefying not nitrate reducing strains the other one comprising all the remaining strains

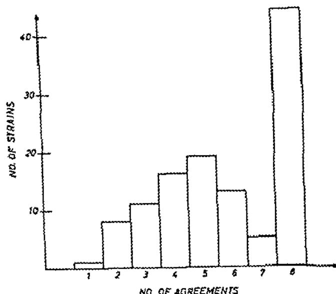


Fig 1

Number of agreements with *Flavobacterium meningosepticum* among 117 aerobic oxidase positive non motile strains concerning sensitivity to eight antibiotics. There is a sharp distinction between strains with the characteristic sensitivity pattern of *Flavobacterium meningosepticum* and the remaining in which sensitivity to the eight antibiotics seems to be distributed at random

strains shown in Table 1 (the five strains identical with *Flavobacterium meningosepticum* were excluded) confirmed that strains with the properties indol production gelatin liquefaction but no nitrate reduction attacked the same carbohydrates as *Flavobacterium meningosepticum* to a higher degree than the remaining strains

The principle of the analysis can be seen from Table 3. The strains were divided into groups I, II and III based on the antibiotics used for isolation. The uppermost line indicates the number of agreements with *Flavobacterium meningosepticum* as regards acid production from the 21 carbohydrates used and the other figures show the number of strains showing the stated number of agreements

Independent of the method of isolation the indol producing gelatin liquefying, non nitrate reducing strains showed more agreement with *Flavobacterium meningosepticum* than the others

Fig 1 shows the antibiotic sensitivity of the 117 strains compared with that of *Flavobacterium meningosepticum*. The abscissa gives the number of agreements with *Flavobacterium meningosepticum* as regards sensitivity to the eight antibiotics mentioned previously and the ordinate the number of strains showing these agreements. Eight agreements correspond to a sensitivity pattern identical with *Flavobacterium meningosepticum* and were found in 44 of the 117 strains. The figure shows a sharp distinction between strains with the peculiar sen

sitivity pattern of *Flavobacterium meningosepticum* and the other strains since only five strains showed seven agreements while the remaining strains showed fewer. The strains with a sensitivity pattern different from that of *Flavobacterium meningosepticum* seem to be distributed at random as regards sensitivity to the eight antibiotics.

DISCUSSION

Strains resembling *Flavobacterium meningosepticum* have previously been isolated from patient specimens (*King* 1963, *Greaves* 1966) but the microorganism has never before been found outside hospital surroundings.

At the Odense County and City Hospital *Flavobacterium meningosepticum* has been isolated frequently within the hospital (*Olsen* 1967 b). Even so, no strain completely identical with *Flavobacterium meningosepticum* was detected among 68,500 patient specimens examined during a period of 4 years, thus demonstrating that the microorganism is rare among patient specimens.

On the basis of the investigations performed and the experiences obtained, a method was elaborated for use in the isolation of the microorganism. Using this method, 72 strains were isolated from 32 soil specimens. Their biochemical properties and sensitivity to antibiotics differed only slightly from those of *Flavobacterium meningosepticum*.

The method described was based on the insensitivity to antibiotics of *Flavobacterium meningosepticum*. Furthermore, the fact that *Flavobacterium meningosepticum* is aerobic, produces indol, liquefies gelatin and does not reduce nitrate is unusual, since as a rule indol producing bacteria reduce nitrate and grow anaerobically (*Leifson* 1960). In addition, aerobic, non-motile, oxidase positive strains with the three biochemical properties mentioned were found to have a tendency to attack the same carbohydrates as *Flavobacterium meningosepticum* (Table 3). However, Table 2 shows that the carbohydrate pattern varies considerably even among these strains.

With a view to discrimination between *Flavobacterium meningosepticum* and resembling bacteria (Flavobacterium sp.), *King* (1963) used six carbohydrates according to the following scheme:

	No. of strains	Gluc.	Xyl.	Mannit.	Lact.	Sucr.	Malt.
Fl. mening. (IIa)	45	+	−	+( )	(+)		+ (late)
Flavobact. sp. (IIb)	26	+(−)	− (weak)	(+)	−	(+)	+(−)

( ) = occasional reaction

If the strains shown in Table 2 are divided on the basis of this scheme there would be 14 *Flavobacterium meningosepticum* strains and 51 Flavobacterium sp while the remaining 72 strains could not be placed Thus a clear cut fractionation based on the carbohydrate pattern does not seem to be possible

King (1965) also described a group of 22 strains (IIf) with biochemical properties like those of *Flavobacterium meningosepticum* except that they did not attack any of the six carbohydrates she used The majority of these strains were isolated from the female genito urinary tract No such strains were found in this study

Indol negative aerobic oxidase positive non motile strains 65 of which were found in the present investigation cannot be classified on the basis of the biochemical reactions used

Several of *King*s strains of Flavobacterium sp were from patient specimens 14 were isolated from blood and spinal fluid No clinical information is available concerning these As mentioned previously similar strains were found in this study but none of the patients showed clinical signs of infection with the microorganism It cannot be determined at present whether these strains may be pathogenic for man Epidemiological investigations can probably be carried out by the selective method described but it may be necessary to use other antibiotics and carbohydrates

SUMMARY

Flavobacterium meningosepticum was isolated from brook water and soil but not from tap water Furthermore it was detected in a specimen from a patient who had no signs of infection with the microorganism

On the basis of the biochemical properties of *Flavobacterium meningosepticum* and its sensitivity to antibiotics and by means of an analysis of other aerobic non motile oxidase positive strains a method was elaborated to be used for the isolation of the microorganism

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Acta path microbiol scandinav 7 323 330 1969

The Department of Otolaryngology University of Lund Lund Sweden

STAPHYLOCOCCAL PRECIPITINS IN NORMAL AND POSTINFECTIOUS HUMAN SERA

By

THORVALD LOFKVIST

Received 13 VII 68

A previous paper (22) described a procedure for separating the antigens of an extract from *Staphylococcus aureus* strain Cowan I The fractions obtained were called A 1 A 2 A 3 B and C Fraction A 1 contains *Jensen* s antigen A (14 15) named protein A by *Oeding* (6) fraction A 3 *Julianelle* s polysaccharide A (17 8 23)

The main purpose of this study was to reveal in normal and pathological human sera the possible presence and frequency of precipitins corresponding to the antigens Since determination of anti-α staphylolysin (ASta) which is now a routine procedure in clinical bacteriological laboratories gives information only about the response to one kind of staphylococcal activity though an important one and sometimes fails to indicate staphylococcal disease (19) it was considered of interest to correlate the results obtained on precipitation with those found on titration of anti α staphylolysin

MATERIAL AND METHODS

Preparations of purified antigen The fractions of antigen (A–1 A–2 A–3 B and C) from S *aureus* type Cowan I Oeding *ab(cl)m* were obtained by gel filtration and zone electrophoresis (22) Saline solutions of the following concentrations were prepared A–1 0 5 mg/ml A–2 1 mg/ml A–3 1 mg/ml B 2 mg/ml and C 40 mg/ml Fraction A–1 and A–3 contain two precipitinogens each while the other three fractions contain only one (23) Fraction C is eluted with an ammonium sulphate peak from a Seph dex G–100 column The used larger amount of fraction C compared with the other fractions reflects the presence of salt

Sera Samples from 141 blood donors served as a normal material The pathological material was made up of 110 sera sent for determination of anti α staphylolysin to the Department of Clinical Bacteriology Malmo General Hospital or to the Department of Medical Microbiology University of Lund[1] They derived from patients infected with S *aureus* (positive cultures) The diagnoses were bronchopneumonia in 26 sinusitis in 10 tonsillitis in 5 pleuritis in 2 osteitis and osteomyelitis in 17 arthritis in 10 pyelonephritis in 17 cystitis in 6 septicaemia in 9 and endcarditis in 8 Among the patients with bone infection 13 had osteitis secondary to open fractures or to osteotomy and 4 had osteomyelitis secondary to septicaemia

The work was supported by grants from *Alfred Österlund s Foundation* Malmö Sweden

[1] Courteously supplied by Professor *Sten Winblad* Malmo and Professor *Rune Grubb* Lund

All of the sera from patients with bone or joint diseases or with septicaemia and most of the other sera refer to 2–3 weeks after the onset of the diseases. Sera from 12 patients with osteomyelitis (19) were courteously placed at our disposal by Professor *Ch. H. Lack*, The Institute of Orthopaedics and The Royal National Orthopaedic Hospital, London.—Merthiolate (1:10000) was added to the sera as a preservative.

Serological technique. The gel precipitin assays were performed with a modified Ouchterlony technique using an immunodiffusion standard set (set 6800 A–7, LBK, Stockholm, Sweden) as in a previous study (24). Each well was filled with 7 µl of reactant. The diffusion was allowed to proceed for 24 hours before the slides were washed and stained with Amido black. Anti-α-staphylolysin (ASta) was determined with the technique devised by *Ingestad & Wanblad* (13).

RESULTS

All 141 *normal* sera precipitated fraction A 1 (protein A) and 11 (7.8 per cent) of these contained also precipitins against fraction A 3 (polysaccharide A) (Table 1). No precipitins against fraction A 2, B and C were found. The majority of the sera (87 per cent) had ASta titres of less than 2.5 IU/ml.

TABLE 1

Precipitins Against S. aureus—Fractions A-1, A-2, A 3, B and C in Sera from Normal Blood Donors

Material	Serum No	ASta IU/ml	Precipitins A-1	A-2	A-3	B	C	Patterns
Blood donors	123	< 2.5	123		8			A 1 130, A 1+A 3 11
Blood donors	18	2.5–10	18		3			
	141		141		11 (7.8%)			

Protein A was also precipitated by all sera from *patients with staphylococcal infections*. Precipitins against A 3 were found in 36 of these sera (30 per cent) and against C in 25 of them (20 per cent), while precipitins against fractions A 2 and B were less common—1.8 and 5.7 per cent respectively (Table 2). In the tests with fractions A 2, A 3, B and C it was especially the sera with high titres of ASta that reacted. Thus antibodies against A 2 and B were found only in sera with ASta of more than 10 IU/ml but precipitins against A 3 and C were also sometimes detected in sera with a low ASta titre. Most of the sera (61 per cent) reacted only with fraction A 1 (Table 3). The patterns A 1 + A 3, A 1 + A 3 + C and A 1 + C were the most common but one among the 10 precipitin combinations observed (Table 3, Fig. 1) occurring in about 16, 11 and 6 per cent respectively. The other 6 combinations (A 1 + A 3 + B + C, A 1 + A 2, A 1 + A 2 + A 3 + B + C, A 1 + A 3 + B, A 1 + B and A 1 + B + C) were rare.

DISCUSSION AND CONCLUSIONS

Various techniques have been used to detect staphylococcal antibodies in human sera (3). The literature on the determination of antitoxins

TABLE 2

Precipitins Against S aureus—Fractions A 1 4- A 3 B and C in Sera f Patients with Staphylococcal Infection

Material	Serum No	ASta IU/ml	A 2	A 3	B	C
Respiratory infections	1	< 2 5				
	18	2 5 10				
	24	> 10	1	4		
	43			7		
Bone and joint infection	9	< 2 5				
	7	2 5-10		2		
	3	> 10		1		1
	33			[illegible]	1	1
Urinary infections	1	< 2 5				
	10	2 5-10				
	12	> 10		1		
	23			3		3
Septicaemia and endocarditis	3	< 2 5				
	2	2 5 10		1		
	12	> 10				1
	17		1	7	6	7
Total	14	< 2 5				
	37	2 5-10		3		
	71	> 10	[illegible]	[illegible]		2
	129		[illegible]	23		1
			[illegible]	37	7	27
		%	1 8	30	7	2
					5 7	29

Respiratory infections Bronchopneumonia 26 Sinusitis 10 T n s l l i t i s 11 ... [illegible]
Bone and joint infections O teitis and O t omyelitis 29 Arth iti s 1 ... u itis 2
Urinary infections Pyelonephriti 17 Cystitis 6
§ All sera precipated fraction A-1

TABLE 3

Precipitin Patterns of Pathological Human S a

ASta	A-1	A-1 + A 3	A 1 + A-3 + C	A 2 + C	Other combinations
< 2 5	9	3	2		
2.5-10	30	6	1		
> 10	34	12	10	7	
	73	1	13		*
Total 1?? sera					

especially anti α staphylolysin is voluminon (e g 1 20 2, 28 29) The diagnostic value of titrations of this antitoxin is debatable (2 3 7) Criticism has been levelled main against the wide ran e of norm values of the antitoxin (3) and a inst the inadequa y of the techni in the investigation of patients in whom the lesion is due to st ph cocci lacking the capacity to produce α toxin (4)

Among the reports of antibod to somatic a tigens only f

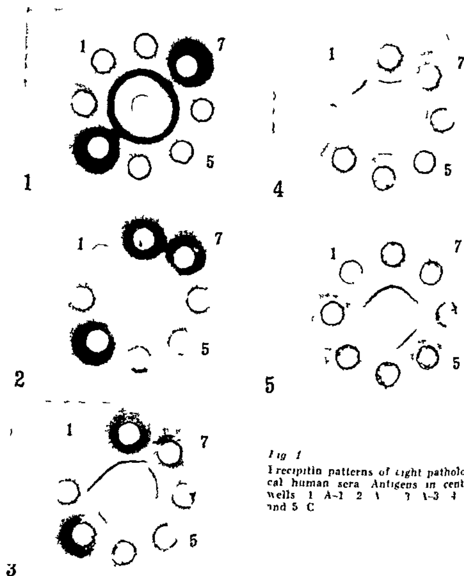


Fig. 1.
Precipitin patterns of eight pathological human sera. Antigens in central wells: 1 A-1, 2 A, 3 A-3, 4 B and 5 C.

with precipitins. In an investigation of cutaneous reactions to polysaccharide A isolated by *Julianelle & Wieghard* (17) *Julianelle & Hartmann* (18) included a study of precipitins in normal and pathological sera. Anti polysaccharide A was found only in patients with severe long standing or generalized staphylococcal infection, i.e. in 1/3 of the pathological sera tested, but normal sera did not contain this antibody. Later *Haukenes* (9) tested some normal human sera for anti polysaccharide A (= teichoic acid antibodies). Like *Julianelle & Hartmann* he did not find any precipitins. Haukenes did not study any pathological sera. Recently *Martin et al.* (25) studied the occurrence of teichoic

and antibodies in sera from normal and infected patients using Ouchterlony gel diffusion plates and the more sensitive Preer method of double diffusion tubes If Ouchterlony plates were used antibodies were demonstrated in 1 per cent of uninfected patients and in 42 per cent of patients who had had staphylococcal infections for more than 30 days In the assays with Preer gel diffusion tubes the corresponding figures were 79 and 96 per cent of the sera

In 1958 *Jensen* (14) observed that normal human sera precipitate an antigen (antigen A) present in the majority of S *aureus*—strains He supposed the antigen might be a polysaccharide (15) but it is now recognized as a protein (protein A) (6 21 22 23) Recent findings (5) indicate that the reaction between protein A and human serum involves a part of the γ globulin molecule other than that in which the active site of acquired antibody is located and the reaction has been called pseudo immune In a later study of the antigenic structure of coagulase positive staphylococci *Jensen* (16) used normal human sera to test unfractionated bacterial extracts in agar gel On the basis of the patterns observed he claimed the existence of at least 8 different precipitinogens in *S aureus* 47 different combinations of the 8 antigens were seen In some experiments Jensen also used sera from patients with staphylococcal infections Such sera contained two or more precipitins more frequently than did sera from healthy persons but they did not offer the possibility of finding any antigen in addition to the eight observed in work with the battery of normal sera Recently a capsular polysaccharide antigen (staphylococcal polysaccharide antigen = SPA) was isolated by *Fischer et al* (4) and *Hofstad* (11) found precipitins against the antigen in immune human sera

The general occurrence of anti A 1 (anti protein A) is in conformity with the results obtained in earlier studies of human sera with protein A (14 21 26) The finding of anti A 3 (anti polysaccharide A s antibodies to teichoic acid) in almost 8 per cent of normal human sera contrasts with the absence of this antibody in such sera studied by *Julianelle & Hartmann* (18) and by *Haukenes* (9) It also differs from the corresponding frequency of 1 per cent reported by *Martin et al* (25) and may exemplify the difficulty of selecting a normal material in a study of serological response to ubiquitous bacteria In the investigation of the pathological sera the results were in good agreement with those obtained by *Julianelle & Hartmann* and by *Martin et al* (presence of antipolysaccharide A in 30 29 and 42 per cent respectively)

As precipitins against A 2 B and C were not found in normal sera their demonstration points to staphylococcal disease Anti C seems to be of special interest since it is found more frequently than anti A 2 and anti B (here in 20 against 1 8 and 5 7 per cent respectively) and unlike anti A 2 and anti B also sometimes in sera with a low titre of ASta It is not yet possible to compare the results obtained with fractions A 2 B and C in this study with those obtained in other investiga

tions because so far the antigens have not been correlated with other precipitinogens described (16) or isolated (4 10 12)

The precipitins were found more frequently in the group of sera with high ASta content Among 81 positive tests with A 2 A 3 B and C 58 occurred in the group with ASta higher than 10 IU/ml (Table 1 and 2) The finding of precipitins (against A 3 and C) also in sera with low or moderately increased titres of ASta (in 12 of 51 pathological sera), indicates that the precipitins technique may sometimes be useful clinically for revealing staphylococcal immunization —Precipitins were detected more frequently in the sera from patients with septicaemia and endocarditis than in the others (Table 2) Thus in the group of sera with ASta>10 IU/ml the following frequency were found 21 precipitins in 12 sera from patients with septicaemia and endocarditis compared with 13 precipitins in 24 sera from patients with respiratory infections 18 precipitins in 23 sera from patients with bone and joint infections and 6 precipitins in 12 sera from patients with urinary infections

The different patterns of precipitins against A 1 A 2 A 3 B and C (Table 3) were not as numerous as those in *Jensen*s study of eight antigens(16) From the results presented here it appears that as a rule staphylococci provoke precipitins to only two or three antigens in combination either because of the more common occurrence of these antigens or because of their greater antigenicity However the technique used does not allow any definite conclusions in this respect—see the different results obtained by *Martin et al* in their study using Ouchterlony plates and Preer tubes (25)

SUMMARY

Sera from 141 blood donors and 122 patients with infections with *S aureus* were examined for their content of precipitins against fraction A 1 A 2 A 3 B and C and for their titre of anti-α staphylolysin (ASta)

All sera contained anti A 1 Among the normal sera 7 8 per cent precipitated fraction A 3 but no precipitins against A 2 B and C were found Among the pathological sera precipitins against A 3 and C were found in 30 and 20 per cent respectively while those against fraction B and A 2 were uncommon 3 7 and 1 8 per cent respectively

In 81 positive tests with the antigens A 2 A 3 B and C 58 occurred in the group with ASta>10 IU/ml In 12 out of 51 pathological sera with a low or moderately increased titre of ASta there were precipitins against A 3 or C or both

61 per cent of the sera reacted only with fraction A 1 The patterns A 1 + A 3 A 1 + A 3 + C and A 1 + C were the most common but one among 10 precipitin combination observed These patterns occurred in 16 11 and 6 per cent respectively

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The Tuberculosis Department and the Biostatistical Department
Statens Seruminstitut Copenhagen Denmark

RAPIDLY GROWING MYCOBACTERIA

Susceptibility to Bacteriophages Reactions in the Amidase Test Production of Acid from Carbohydrates and Growth at Various Temperatures

By

I BAESS and M WEIS BENTZON

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The aim of the present work is to examine the suitability of mycobacteriophages in the classification of rapidly growing mycobacteria

During recent years *Kappler* (1961) *Rodda* (1964) *Tokunaga et al* (1965) and *Juhasz & Bonicke* (1965) have described phage typing of rapidly growing mycobacteria The results of their studies as well as own experience have shown that if the phages are species specific they do not lyse all strains within the species Only within *M phlei* are there a few species specific phages which lyse all strains (*Rodda* 1964 *Juhasz & Bonicke* 1965)

The material in this study consists of both previously classified and unclassified strains In order to obtain further characterization all strains were examined for their acylamidase reactions (*a m Bonicke*) for their ability to produce acid from carbohydrates and for their capability of growth at 45 and 52 C The quantitative analysis of these results forms the basis for evaluation of the specificity of mycobacteriophages and their suitability for the identification of the classified strains of rapidly growing mycobacteria The analysis proved also to be a valuable asset in identifying the unclassified strains

MATERIAL

Bacterial strains Table 1 gives a survey of the 76 strains examined and their origin Five were variants of one of the others and one (serial No 27) seemed to be an admixture with another strain (serial No 28)

Forty three strains had a species designation when received These were 9 *M phlei* 11 *M smegmatis* 16 *M fortuitum* and 7 *M vaccae* Among these three strains of *M fortuitum* (serial Nos 9 11) were isolated from Danish patients without being of pathogenic significance (*Engbæk* 1967 *Engbæk & Magnusson* 1961)

The majority of the 33 unclassified strains were isolated at the Tuberculosis Department of Statens Seruminstitut Twenty one were from sputum (two have been described previously (serial Nos 57 and 58) (*Engbæk & Magnusson* 1961 *Magnusson et al* 1961) five were from soil from poultry runs and seven were received from other laboratories

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Requests for reprints Dr *Inga Baess* The Tuberculosis Department Statens Serum Institut Amager Boulevard 80 2300 Copenhagen S Denmark Tel AS 2817

Morphology

Since the colony morphology was not uniform for strains within the same species and furthermore a few strains contained variants this aspect was not used for characterization of the strains The majority of the *M phlei* were coiled yellow to orange and rough The appearance of the *M smegmatis* was very variable They were yellowish brown and both smooth and rough strains were seen Most of the *M fortuitum* were coiled or berry shaped yellowish brown and smooth Apart from the two rough variants the *M vaccae* were flat dome-shaped, yellowish and smooth The unclassified strains showed considerable variation in morphology

Microscopy

M fortuitum and *M vaccae* consisted of completely acid fast rods and a few of the *M phlei* and *M smegmatis* were partly decolorized The unclassified strains showed acid fast rods ranging from very short to long Two of them (serial Nos 52 and 53) were not acid fast and had a polymorphous appearance

Ability to Grow at Various Temperatures

All strains grew at 37 C At 45 C all the *M phlei* and *M smegmatis* showed growth as well as two *M fortuitum* and a few of the unclassified strains Only the nine *M phlei* strains grew at 52 C

Amidase Test

The results are given in Table 2 The first part of the table comprises the classified strains and shows how many strains within each species react to the different amides The next section of the table shows the unclassified strains with the same pattern as a classified species while in the lower part the other unclassified strains are divided according to their pattern Reactions of $> 2\ \gamma/ml$ are recorded as positive and those $\leqq 2\ \gamma/ml$ as negative It will be seen that there is a clear difference in the results as regards the first three species *M phlei* *M smegmatis* and *M fortuitum* Particularly the reactions to amides 11 (formamide) 12 (propionamide) and 13 (butyramide) show a characteristic pattern *M vaccae* resembles *M smegmatis* Among the unclassified strains only a few show results corresponding to those of the known species Two resemble *M phlei* (serial Nos 71 and 75) one resembles *M smegmatis* (serial No 28) and three resemble *M fortuitum* (serial Nos 27 50 and 77)

Production of Acid from Carbohydrates

All strains grew well in Hugh & Leifson s medium The results are shown in Table 3 The four species showed different patterns In these

TABLE 2
Amidase Reactions of 76 Rapidly Growing Mycobacteria

Bacteria	No of strains	Amides 1	2	3	4	5	6	7	8	9	10	11	12	13
M phlei	9	0	0	3	0	9	9	0	0	0	0	9	0	1
M smegmatis	11	9	11	7	11	11	11	7	1	11	6	11	11	11
M fortuitum	16	16	0	3	0	1	2	0	0	0	0	16	16	0
M vaccae	7	7	7	0	7	7	7	2	0	4	0	7	7	7
Unclass (as *M phlei*) (Nos 71 75)	2	0	0	1	0	[illegible]	[illegible]	0	0	0	0	2	0	0
Unclass (as *M smegmatis*) (No 28)	1	1	1	1	1	1	1	1	0	1	0	1	1	1
Unclass (as *M fortuitum*) (Nos. 27 50 77)	3	3	0	[illegible]	0	0	0	0	0	0	0	3	3	0
Unclassified	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0	0	10	0	0
"	3	0	0	3	0	0	0	0	0	0	0	3	0	0
	1	0	0	1	0	0	0	0	1	0	0	1	0	0
	1	0	0	1	0	0	0	0	0	1	1	1	0	0
	1	0	0	1	0	1	0	0	1	0	1	1	0	0
	1	1	0	0	0	0	1	0	0	1	0	1	1	0
	1	1	0	0	0	0	1	0	0	0	0	0	1	0
	1	0	1	0	1	1	1	0	0	0	0	1	1	1
"	1	1	1	1	0	1	1	1	0	1	1	1	1	1
	1	1	1	1	0	1	1	0	1	1	1	1	1	1
	1	0	0	1	0	1	1	0	0	0	0	1	1	1
	1	0	0	1	0	1	1	0	0	0	0	0	0	1
	2	0	0	0	0	[illegible]	[illegible]	0	0	0	0	0	0	0

Notes
1 = acetamide 2 = benzamide 3 = carbamide 4 = isonicotinamide 5 = nicotinamide 6 = pyrazinamide 7 = salicylamide 8 = allantoin 9 = succinamide 10 = malonamide 11 = formamide 12 = propionamide 13 = butyramide

experiments there was a difference between *M smegmatis* and *M vaccae*, since only *M smegmatis* formed acid from galactose and sorbitol One of the unclassified strains showed the same result as *M smegmatis* (serial No 28) and three the same result as *M fortuitum* (serial Nos 27 50 and 77) The remainder gave other patterns

Susceptibility to Mycobacteriophages

The results of the experiment with ten phages are shown in Table 4 Since the majority of these phages were *not* species specific six were selected in order to obtain a division of the bacterial strains which is in better agreement with the accepted species *viz* D11 Bk, D4 Bk, B5 and D30

The results as regards the classified strains are shown in Table 5 It will be seen that D11 is almost species specific for *M phlei* and Bk and D4 for *M smegmatis* Bk and B5 are not species specific but Bk lysed all the *M fortuitum* strains

TABLE 3
Acid Production of Carbohydrates by 76 Rapidly Growing Mycobacteria

Bacteria	No of strains	Gl	Rh	Xy	Ar	Ga	Mt	So	It	Du	La	Ma	Tr
M phlei	9	9	0	4	5	8	9	9	0	0	0	2	7
M smegmatis	11	11	11	10	11	11	9	11	11	2	0	3	8
M fortuitum	16	16	0	0	0	1	0	0	0	0	0	0	0
M vaccae	7	[illegible]	5	6	7	0	7	0	6	0	0	1	0
Unclass (as *M smegmatis*) (No 28)	1	1	1	1	1	1	1	1	1	1	0	0	1
Unclass (as *M fortuitum*) (Nos 27 50 77)	3	3	0	0	0	0	0	0	0	0	0	0	0
Unclassified	7	0	0	0	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	8	0	0	0	0	0	0
	1	0	0	1	0	0	0	0	0	0	0	0	0
	2	0	0	2	2	0	0	0	0	0	0	0	0
	1	1	0	0	1	0	1	0	0	0	0	0	0
	1	1	0	0	0	0	1	1	0	0	0	0	0
	2	0	0	0	0	0	2	0	2	0	0	0	0
	1	1	0	1	0	0	1	1	0	0	0	0	0
	1	1	0	0	0	0	1	0	1	0	0	0	0
	1	1	0	0	0	0	1	0	1	0	0	0	1
	1	1	0	0	1	0	1	0	1	0	0	0	1
	1	1	0	1	1	1	1	0	0	0	0	0	0
	1	1	1	1	0	0	1	1	0	0	0	0	0
	1	1	1	1	1	1	1	1	0	0	0	0	0

Carbohydrates
Gl = glucose Rh = rhamnose Xy = xylose Ar = arabinose Ga = galactose Mt = mannitol So = sorbitol It = inositol Du = dulcitol La = lactose Ma = maltose Tr = trehalose

The patterns of the four species differ from each other One strain of *M vaccae* was lysed by more phages than the others This was a rough variant of one of the five strains lysed by B5 on the basis of the other tests it resembled the smooth strain completely

None of the unclassified strains (Table 6) showed the same lysis pattern as *M phlei* One was lysed in the same way as *M smegmatis* (serial No 28) twelve as *M fortuitum* (incl serial Nos 27 50 and 77) and one as *M vaccae* (serial No 60) Two of the 76 strains were lysed by D30 (serial Nos 52 and 53)

Quantitative Analysis of Results

The results of growth at 45 and 52 C the reactions to the thirteen amides and to the twelve carbohydrates were subjected to quantitative analysis (i e 27 characteristics in all) in order to ascertain whether *there was homogeneity within the individual species* of the classified strains

The results are recorded as positive or negative to each of the 27

TABLE 4

Susceptibility to Mycobacteriophages of 76 Rapidly Growing Mycobacteria

Bacteria	No of strains	Mycobacteriophages D4	Bk$_1$	D12	F21/1 AH	D11	B10	BK	D30	B5	D32
M phlei	9	0	0	0	0	9	8	0	0	1	0(1)
M smegmatis	11	6	11	11	9(1)	0	6	6	0	6	6
M fortuitum	16	0	0	1	0	0	1(1)	16	0	2(6)	1(1)
M vaccae	7	0(1)	0	0	0	1	0(1)	1	0	6	0(1)
Unclass (as *M smegmatis*) (No 28)	1	1	1	0	1	0	1	1	0	1	1
Unclass (as *M fortuitum*) (incl Nos 27 50 77)	12	0	0	2	0	0	2	12	0	0(1)	0(1)
Unclassified	7	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0(2)	0
	2	0	0	0	0	0	0	0	2	0	0
	1	0	0	0	0	0	0	0	0	0	1
	2	0	0	0	0	0	2	0	0	0	2
	2	0	0	0	0	0	2	0	0	1(1)	1(1)
	1	0	0	0(1)	0	0	0(1)	0	0	0	0
	1	0(1)	0	0(1)	0	0	1	0	0	1	1
"	1	0	1	1	0	0(1)	1	1	0	1	1
"	1	1	0	0	0	0(1)	1	0(1)	0	1	1

Figures in brackets represent variable results

TABLE 5

Susceptibility to Six Mycobacteriophages of 43 Classified Strains of Rapidly Growing Mycobacteria

Classified strain	No of strains	Mycobacteriophages D11	BK	D4	BK	B5	D30
M phlei	8	+	—	—	—	—	—
	1	+	—	—	—	+	—
M smegmatis	5	—	+	—	—	—	—
	6	—	+	+	+	+	—
M fortuitum	8	—	—	—	+	—	—
	7	—	—	—	+	+	—
	6	—	—	—	+	V	—
M vaccae	1	—	—	—	—	—	—
		—	—	—	—	+	—
	1	+	—	V	+	+	—

+ = lysis — = no lysis V = variable result

TABLE 6

Susceptibility to Six Mycobacteriophages of 33 Unclassified Strains of Rapidly Growing Mycobacteria

Unclassified strains	No of strains	Mycobacteriophages					
		D11	Bk_1	D4	Bk_4	B5	D30
As M smegmatis	1	—	+	+	+	+	—
As M fortuitum	11	—	—	—	+	—	—
	1	—	—	—	+	V	—
As M vaccae	1	—	—	—	—	+	—
Other patterns	11	—	—	—	—	—	—
	2	—	—	—	—	—	+
	3	—	—	—	—	V	—
	1	—	—	V	—	+	—
	1	V	—	+	V	+	—
	1	V	+	—	+	+	—

+ = lysis — = no lysis V = variable result

TABLE 7

Patterns of Four Species of Rapidly Growing Mycobacteria

Bacteria		*M phlei*	*M smegmatis*	*M fortuitum*	*M vaccae*
Growth	45 C	+	+	—	—
	52 C	+	—	—	—
Amidase reactions	1	—	+	+	+
	2	—	+	—	+
	3	V	V	—	—
	4	—	+	—	+
	5	+	+	—	+
	6	+	+	—	+
	7	—	V	—	—
	8	—	—	—	—
	9	—	+	—	V
	10	—	V	—	—
	11	+	+	+	+
	12	—	+	+	+
	13	—	+	—	+
Acid production	*Gl*	+	+	+	+
	Rh	—	+	—	+
	Xy	V	+	—	+
	Ar	V	+	—	+
	Ga	+	+	—	—
	Mt	+	+	—	+
	So	+	+	—	—
	It	—	+	—	+
	Du	—	—	—	—
	La	—	—	—	—
	Ma	—	—	—	—
	Tr	+	+	—	—

+ = positive results for ≧ 70 per cent of strains
— = negative results for ≧ 70 per cent of strains
V = positive and negative results in less than 70 per cent

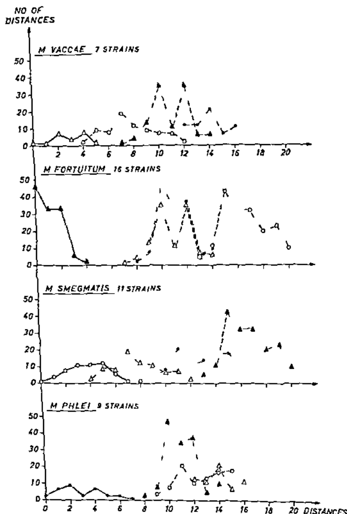


Fig 1

Distances between individual strains of the four species *M phlei M smegmatis M fortuitum* and *M vaccae*
● *M phlei* ○ *M smegmatis* ▲ *M fortuitum* △ *M vaccae*
——— Distances between strains belonging in one species
- - - - - Distances to strains belonging in other species

characteristics Comparison between the various strains is made pair wise the number of divergencies between strains being counted for each pair This number of divergencies is called the distance between the two strains

Fig 1 shows the distribution of the distances between the individual strains for each of the four species together with the distribution of the distances from these strains to those belonging in the three other species It will be seen for example that as regards *M phlei* the greatest

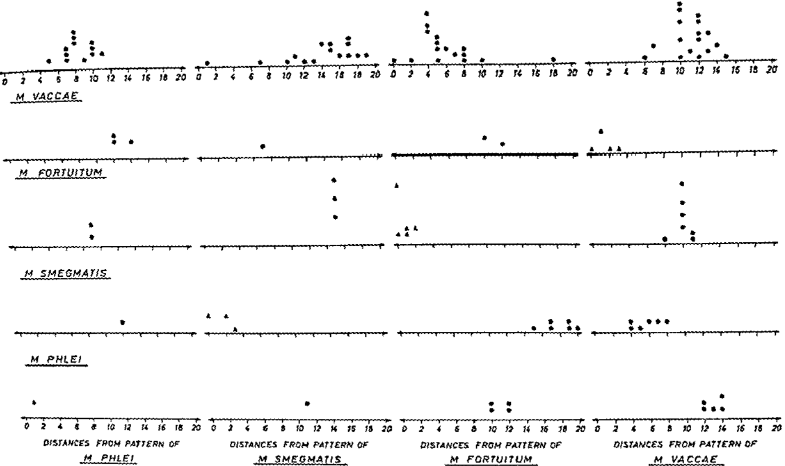


Fig 2

Distances from individual strains to patterns of *M phlei M smegmatis M fortuitum* and *M vaccae* Each symbol represent one strain

distance (i e 7) between two M phlei strains is less than the smallest distance (i e 8) between the M phlei strains and those of another species Distance 8 is found between M phlei and three M fortuitum strains The greatest distance between the M fortuitum strains is 4 and the smallest distance to a strain of another species is 7 Only between the M smegmatis and M vaccae strains do the distributions of distances overlap

TABLES 8 a—d

Qualitative Evaluation of Rapidly Growing Mycobacteria Deviating from Their Pattern

Table 8 a *M phlei*

Serial No	Distance	Temp	Amides	Carbohydrates
37	1			Tr
38	3			Ga Ma Tr
78	1			Ma
79	1		13	

Table 8 b *M smegmatis*

Serial No	Distance	Temp	Amides	Carbohydrates
17	1			Ma
18	1			Ma
19	2		1	Du
22	2			Du Ma
23	2			Mt Tr
24	2		1	Tr
25	1		8	
26	3			Xy Mt Tr
28	1			Du

Presumably *M smegmatis*

Table 8 c *M fortuitum*

Serial No	Distance	Temp	Amides	Carbohydrates
3	2	45 C	3	
4	1			Ga
8	2		5 6	
11	1		6	
12	1		3	
13	2	45 C	3	
27	[illegible]	4 C	3	
50	1		3	
57	3		1 12	Gl
64	3		1 12	Gl
77	0			

Presumably *M fortuitum*

Table 8 d *M vaccae*

Serial No	Distance	Temp	Amides	Carbohydrates
30	1		7	
32	1			Ma
33	1		7	
34	1			Xy
35	2			Gl Rh
36	3			Gl Rh R

For names of amides and carbohydrates see Tables 2 and 3

A pattern of each of the species was then established so that a result shown by ≧ 70 per cent of the strains is regarded as typical for that species For instance, when 70 per cent or more of the strains of one species ferment a certain carbohydrate that characteristic is regarded as positive for the pattern of the species If both positive and negative results are found in less than 70 per cent the designation variable is used The patterns of the four species according to this definition are shown in Table 7

The distance between a strain and a pattern is calculated in the same way as the distance between the strains except if the individual characteristics of the pattern are variable in which case no difference is recorded

The distances between the classified and the unclassified strains and the patterns of the four species can be seen from Fig 2 The greatest distance between the classified strains and their own pattern is 3 (for *M fortuitum* only 2) The smallest distance from a classified strain to another pattern is 4 viz the distance between *M smegmatis* and the *M vaccae* pattern and vice versa It was also the *M vaccae* and *M smegmatis* strains that showed overlapping in Fig 1 Otherwise the shortest distance between a strain and a heterologous pattern is 8

It will also be seen from Fig 2 that six of the 33 unclassified strains show a distance of 3 or less between them and one of the patterns Since the distance between a classified strain and its pattern can be up to 3 these six strains may be presumed to belong to the corresponding species

As basis for a *qualitative evaluation of the strains* Tables 8a d show the characteristics by which the strains differ from their pattern As regards the classified strains only those which deviate from their pattern are shown and only the diverging characteristics are indicated It will be seen that the strain thought to be *M smegmatis* (serial No 28) differs from the smegmatis pattern since it produced acid from dulcitol Two of the designated *M smegmatis* strains also produced acid from dulcitol As regards the five strains thought to be *M fortuitum* (serial Nos 27 50 57 64 and 77) it will be seen that one is in com

plete agreement with the fortuitum pattern while two show the same deviations as some of the classified *M fortuitum* strains The deviations with serial Nos 57 and 64 occur in characteristics in which none of the classified *M fortuitum* strains show divergency

In order to identify an unclassified strain the criterion used in this study is that it should not deviate from the corresponding pattern in more than three characteristics and that these possible divergencies should occur in characteristics in which some of the classified strains also can deviate from the pattern Thus the strain with serial No 28 can be considered to be *M smegmatis* and only the strains with serial Nos 27 50 and 77 would be classified as *M fortuitum*

Evaluation of Bacteriophages

The quantitative analysis of the results obtained by the methods used has shown that relatively good distinction between the four species *M phlei*, *M smegmatis*, *M fortuitum* and *M vaccae* can be obtained It is therefore possible to utilize the material for an evaluation of the usefulness of bacteriophages in the classification of rapidly growing mycobacteria Six bacteriophages out of the ten available were selected

As mentioned under the results of phage typing and as seen from Tables 5 and 6 none of the other classified or the unclassified strains were lysed as *M phlei* The *M phlei* pattern was therefore considered to be specific Phage D11 lysed *M phlei* almost exclusively but is not 100 per cent specific for that species The pattern of *M smegmatis* is also specific in this material One unclassified strain (serial No 28) was lysed with the same pattern as that of *M smegmatis* As mentioned above this strain could also be classified as *M smegmatis* on the basis of the quantitative analysis of the other tests Phages Bk_3 and D4 are each almost specific The use of phages gives a very clear distribution of *M smegmatis* into two subspecies since five of the eleven strains could be lysed by Bk_3 only while the other six were also lysed by D4 Bk_4 and B5 The pattern of *M fortuitum* is not specific twelve of the unclassified strains being lysed in the same way only three of these (serial Nos 27 50 and 77) could be identified as *M fortuitum* on the basis of the quantitative analysis Bk_4 and B5 are not specific for *M fortuitum* but Bk_4 lysed all the strains of that species The pattern of *M vaccae* is not specific In this study B5 is not specific for *M vaccae* which is in contrast to the findings by *Bonicke & Juhasz* (1964) and *Juhasz & Bonicke* (1965) B5 lysed six of the seven strains One of the unclassified strains (serial No 60) was lysed as *M vaccae* but otherwise it does not resemble that species

At the present stage it must be concluded that bacteriophages alone cannot be used to classify rapidly growing mycobacteria The method is quick to perform and can be used for a screening of unclassified strains If a strain is lysed with the same pattern as that of *M phlei* or

M smegmatis, it is almost certain that it belongs in the species in question If it is lysed with the same pattern as that of *M fortuitum* or *M vaccae*, further examination is necessary before it can be identified as belonging in one of these species

DISCUSSION

In the present study it was taken for granted that the available strains of *M phlei*, *M smegmatis* *M fortuitum* and *M vaccae* belonged in and were representative of the species concerned In the quantitative analysis it was found possible to obtain an acceptable differentiation of the four species by means of distance determinations based on the characteristics employed (Fig 1) A positive outcome of that method was essential in order to see whether the classified mycobacteria in this study could be used in an attempt to identify unclassified strains and to judge the value of bacteriophages in the classification of rapidly growing mycobacteria

Patterns of a group of strains belonging in a species can be established in many ways In the literature the limit used for specifying a positive result for a characteristic varies between 100 per cent and 50 per cent

The patterns given by ≧ 70 per cent of the strains provide a better differentiation of the four species than the difference between the individual strains The choice of 70 per cent is based on an estimate of what would be practicable, judged by the results obtained *Tsukamura* (1967) also regards a characteristic as positive or negative when ≧ 70 per cent of the strains show the same result These patterns were also useful for an identification of unclassified strains but this was only possible by qualitative evaluation of the selected strains in comparison with the patterns concerned (Tables 8a–d)

A few of the characteristics included in the quantitative analysis were different from those reported in the literature and these will therefore be discussed here

The amidase patterns are generally in agreement with *Bonicke's* reports but there are divergencies with regard to the reactions to carbamide allantoin and acetamide In the present study the carbamidase activity was very low which is also in contrast to the findings by *Juhlin* (1967) *Tsukamura* (1967) *Schneidau* (1963) and *Mulder-de Jong* (1963) This low activity cannot be explained The allantoinase activity is also low (*M fortuitum* *M vaccae*) A similar result was found by *Juhlin* in the case of *M fortuitum* However the acetamidase activity was very strong both in *M smegmatis* and *M vaccae* According to *Juhlin* *Schneidau* and *Tsukamura* this was also found to apply to *M smegmatis*

Even though the acid production tests were not carried out in the same medium the results obtained in this study are generally the same

as those found by *Gordon & Smith* (1953 1955) *Gordon & Mihm* (1959) and *Juhlin* (1967) The main difference is that the majority of the *M smegmatis* in this work did not produce acid from dulcitol and that the *M fortuitum* gave no reaction to trehalose In contrast to the findings by *Bonicke & Juhasz* (1964) *M vaccae* did not produce acid from trehalose either

Bacteriophages are not included in the quantitative analysis of the results since that analysis was used as basis for an evaluation of the value of phage typing Phage typing of mycobacteria has hitherto been very difficult to perform both because some species are resistant to the phages isolated up to now and because the existing phages possess marked non specificity or very narrow host ranges The best possibility of establishing a phage pattern seems to exist within Runyon s Group IV During recent years four works have been published concerning this aspect (*Kappler* 1961 *Rodda* 1964 *Tokunaga et al* 1965 *Juhasz & Bonicke* 1965) *Kappler* reported that *M smegmatis* could be divided into two subspecies by means of phages as was also found in this study *Rodda* and *Murohashi* found that D11 was specific for *M phlei*, and *Rodda* reported lysis of 100 per cent of the strains D4 was specific for *M smegmatis* according to *Murohashi* but not according to *Rodda* *Juhasz* examined the same four species as those in this study and also compared the phage results with those of amidase tests However apart from B5 he used other phages He found a pattern specific for *M phlei* and *M vaccae* In contrast to our findings B5 was specific for *M vaccae* but lysed only 84 per cent of the strains The patterns of *M fortuitum* and *M smegmatis* were not specific

In the present study the patterns of *M phlei* and *M smegmatis* are specific and apparently all strains of these two species can be identified by means of the phages used However the pattern of *M fortuitum* is not specific

Twenty nine of the 33 unclassified strains could not be identified as belonging in the four known species It can be seen from distance tables (not included in this work) that many of them have very small mutual distances Three of the strains are identical and may thus be supposed to belong in the same unknown species They do not grow at 45 or 52 C they react only to form amide form acid with mannitol only and are lysed by BK, However until further examples of such strains are found no new species designation can be suggested Two of the unclassified strains (serial Nos 52 and 53) were not acid fast and these are probably not mycobacteria The colonies were reddish in colour They were the only two strains that were lysed by phage D30 which is said to lyse nocardia but not mycobacteria It is probable that these two strains belong in the nocardia group This is in agreement with the observations of *Froman* (1961) and *Bonicke & Juhasz* (1965)

SUMMARY

Forty three classified and 33 unclassified rapidly growing mycobacteria were examined with a view to their ability to grow at 45 and 52° C their reaction to thirteen amides their ability to produce acid from twelve carbohydrates and their sensitivity to ten bacteriophages

A quantitative analysis of the 27 first mentioned characteristics was made in the form of distance tables A definition of a typical pattern of the four species *M phlei M smegmatis, M fortuitum* and *M vaccae* was established and a comparison made between the individual strains and the patterns concerned It was shown that it was possible to group the classified strains into their respective species by means of these methods

On the basis of the distances between the unclassified strains and the species patterns and a qualitative comparison with the patterns it was found that only four of the 33 unclassified strains could be allocated to the known species The remainder of the strains must belong in other species Many of the unclassified strains had small mutual distances and three resembled each other completely However the number of strains was too small for new species designations to be established on that basis

By means of six bacteriophages it was possible to set up a schedule for phage typing of rapidly growing mycobacteria to be used in a screening of unclassified strains The patterns of *M phlei* and *M smegmatis* are specific for the strains examined in this study

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SUMMARY

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A quantitative analysis of the 27 first mentioned characteristics was made in the form of distance tables A definition of a typical pattern of the four species *M phlei*, *M smegmatis*, *M fortuitum* and *M vaccae* was established and a comparison made between the individual strains and the patterns concerned It was shown that it was possible to group the classified strains into their respective species by means of these methods

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Each bottle was inoculated with 10 ml of *B pertussis* (strain 18-530 Grace Eldering Division of Laboratories Michigan Department of Health) suspension and incubated for 2 days at 35 C The cells from 20 Roux bottles were harvested from the agar surface pooled and adjusted to 200 ml with 0 9 per cent sodium chloride The resulting suspension was diluted until the turbidity equaled that of the WHO 2nd International Opacity Reference Preparation (10 IU of opacity per ml) when measured with Beckman DB at 530 mμ The growth was expressed as International Opacity Units per ml calculated from the dilution

The results are shown in Table 1 When cysteine was omitted from the culture medium a cell yield 18 per cent smaller than that provided with the routine procedure (750 IU of opacity/ml) was obtained Replacement of cysteine by an equal amount of pyruvic acid resulted in a crop that was 87 per cent of the one in the routine procedure However if pyruvic acid was sterilized by filtration the growth of *B pertussis* was practically equal to the growth on the routine medium Even better results were obtained when 300 mg per litre of pyruvic acid was added

When the loss of sulphur with cysteine was compensated by adding inorganic sulphate together with pyruvic acid to the culture medium the cell yields were practically equal or larger than those obtained with the routine procedure With 300 mg per litre of pyruvic acid and 35 mg per litre of sodium sulphate sterilized by filtration about 33 per cent more cells were obtained than on the medium of *Holt* (1962)

Thus the enhancement of the growth of *B pertussis* by cysteine is a least partially due to the formation of pyruvic acid during autoclaving Whether the apparent growth promoting effect of sodium sulphate in the presence of pyruvic acid depends on the utilization of inorganic sulphur by *B pertussis* remains to be studied

The nature of the inhibitory substance formed during autoclaving of the culture media with pyruvic acid is not known It may be related to the substance indicated by *Rowatt* (1957) She studied the effect of autoclaved cysteine on the growth of *B pertussis* and emphasized the appearance of a growth inhibiting agent which was believed to be an organic peroxide

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BRIEF REPORTS

THE EFFECT OF PYRUVIC ACID ON THE GROWTH OF *BORDETELLA PERTUSSIS*

By *Veli Kauppinen* and *Lilian Hjerp*

According to *Jebb & Tomlinson* (1957) the sulphur requirement of *Bordetella pertussis* was met by cysteine cystine or glutathione whereas methionine and inorganic sulphate were ineffective The effect of cysteine was attributed to the formation of cystine during autoclaving For the production of pertussis vaccine cysteine is routinely added to the culture medium of *B pertussis* (*Holt* 1962)

On the other hand, it has been found (*Kauppinen* 1968) that cysteine is partially converted to pyruvic acid during autoclaving of a yeast culture medium Therefore it was of interest to study whether autoclaving of *B pertussis* culture medium (*Holt* 1962) results in formation of pyruvic acid and what is the effect of pyruvic acid on the growth of *B pertussis*

Pyruvic acid was determined in the autoclaved *B pertussis* culture medium according to *Bucher et al* (1965) From 30 mg of added cysteine 10 mg of pyruvic acid was formed

The growth experiments were performed as follows For each test, twenty 1 litre Roux bottles were used with 150 ml of the basic agar medium in each The composition of the basic medium was that used by *Holt* (1962) except that cysteine was omitted Before the bottles were sterilized by autoclaving cysteine pyruvic acid or pyruvic acid with sodium sulphate was added as indicated in Table 1 In some experiments pyruvic acid was sterilized by filtration and added aseptically to the basic medium in Roux bottles after autoclaving (see Table 1)

TABLE 1

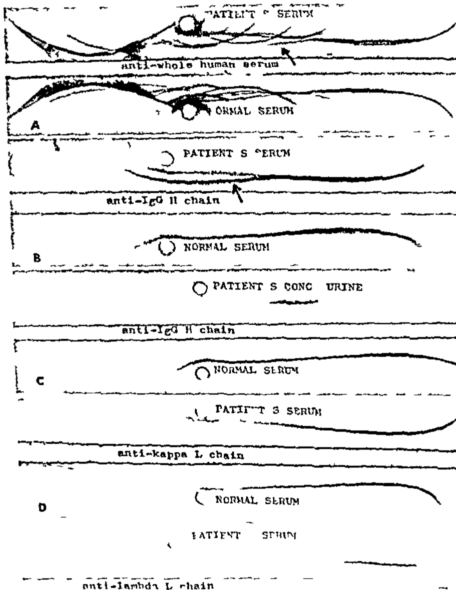
The Effect of Pyruvic Acid on the Growth of Bordetella pertussis

Medium composition	Sterilization process of added nutrient	Growth after two days (International Opacity Units/ml)
Basic medium	Autoclaving	670
+ 30 mg/l cysteine†	Autoclaving	750
+ 30 mg/l pyruvic acid	Autoclaving	650
+ 30 mg/l pyruvic acid	Membrane filtration	703
+ 300 mg/l pyruvic acid	Autoclaving	670
+ 300 mg/l pyruvic acid	Membrane filtration	900
+ 30 mg/l pyruvic acid + 35 mg/l Na_2SO_4	Autoclaving	760
+ 30 mg/l pyruvic acid + 35 mg/l Na_2SO_4	Membrane filtration	900
+ 300 mg/l pyruvic acid + 35 mg/l Na_2SO_4	Autoclaving	700
+ 300 mg/l pyruvic acid + 35 mg/l Na_2SO_4	Membrane filtration	1000

Corresponds to the medium described by *Holt* (1962) except that cysteine is omitted

† Identical to the medium of *Holt* (1962) Used routinely in our laboratory

Received 9 xii 68 from *The State Serum Institute Helsinki Finland*
We wish to thank Miss *Asta Holmstrom* and Mrs *Ensi Tuomela* for their skillful technical assistance


PATIENT S SERUM
anti-whole human serum
NORMAL SERUM
A
PATIENT S SERUM
anti-IgG H chain
NORMAL SERUM
B
PATIENT S CONC URINE
anti-IgG H chain
NORMAL SERUM
C
PATIENT S SERUM
anti-kappa L chain
NORMAL SERUM
D
PATIENT SERUM
anti-lambda L chain

Acta path microbiol scandinav 75 350-35? 1969

TWO CASES OF IgG HEAVY CHAIN DISEASE

By Odd Wager Jorma A Räsunen Laila Lindeberg and Veikko Makela

The M components in IgG myeloma are complete IgG molecules consisting of two light (L) and two heavy (H) polypeptide chains In IgG heavy chain disease the M component is devoid of L chains and related to the Fc fragment of the H chain and it is present both in serum and urine

Since 1964 a total of 8 cases of IgG heavy chain disease have been published (1 2 3 4 6) The clinical picture is characterized by lymphadenopathy hepatosplenomegaly erythema and oedema of the soft palate recurrent bacterial infections particularly pneumonias but not by sceletal involvement Six of the 8 patients have died

In the present preliminary communication some clinical and immunologic data of two additional patients are reported

A woman of 65 with 20 years history of Sjögren's syndrome and of pulmonary tuberculosis in remission since 1965 developed in 1968 prolonged fever and swelling of the right supraclavicular nodes Biopsy (Fig 1) showed abundant atypical plasmacytosis in different stages of maturity Russell bodies were also seen

Immunoelectrophoresis of her serum (Fig 2) revealed an anodic broad deviation of the IgG line detectable with sera containing antibodies to IgG H chain but not with those specific for L chain Routine tests did not detect proteinuria In concentrated urine however an M component of similar mobility and immunologic rea

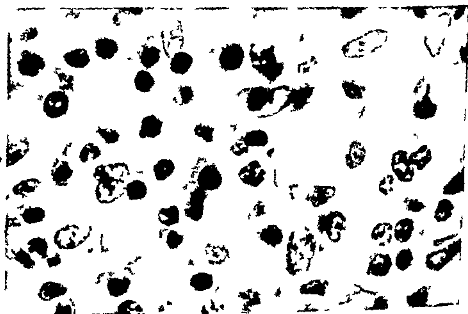


Fig 1

Received 20 xii 68 from the Municipal Bacteriological Laboratory Aurora Hospital Helsinki Finland

This study was supported by grants from the Sigrid Juselius Foundation Finland

The anti IgE serum was obtained through the courtesy of dr H Bennich

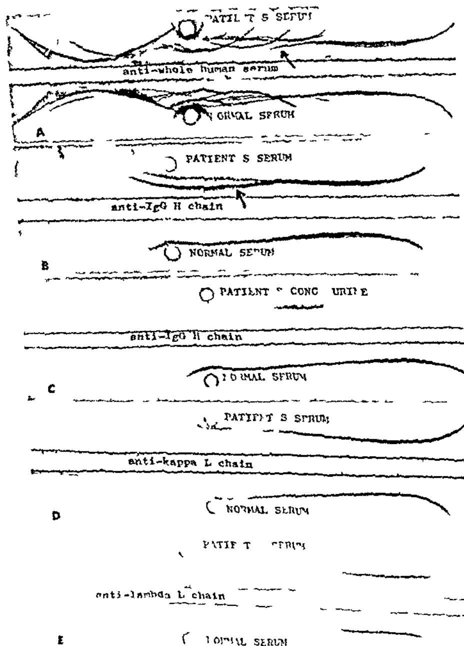


Fig 2

Immun clectr ph re i f the serum and urine of patient 1

tivity was found on immunoelectrophoresis and on agar and cellulose acetate electrophoresis a corresponding narrow band was seen. No narrow bands suggestive of M components were found in serum

These findings were considered strong evidence of IgG heavy chain disease and search for additional cases was initiated Immunoelectrograms of sera from 173 patients with IgG M components were reviewed and 12 sera with anodic broad deviations were selected for re evaluation One of them yielded a similar immuno electrophoretic pattern as that described above

This male patient of 63 had since 1965 been treated 6 times at the Aurora hospital for pneumonias once presenting a positive blood culture for pneumococci After his latest hospitalization in January 1968 he had remained in fairly good general condition He had bronchiectasias splenomegaly but no lymphadenopathy

The M components were isolated from serum by ammonium sulphate at half saturation followed by Pevikon zone electrophoresis using veronal buffer pH 8 6 ionic strength 0 075 and by gel filtration on Sephadex G-150 in 1 N propionic acid and from urine by ammonium sulphate followed directly by gel filtration

The urine M components gave $S_{20\,w}$ values of 4 0 and 2 8 their hexose contents were 10 2 and 14 1 per cent respectively

In Ouchterlony analysis the components gave reactions of identity with each other when *tested against anti IgG H or -Fc* whereas *both gave reaction of partial* identity with IgG when tested against anti IgG H and reaction of identity when tested against anti Fc This pattern suggests absence from the M components of some H chain epitopes present on the whole IgG H chain (Fd epitopes?) Neither of the two M components reacted with antisera to Fab L chains IgM IgA IgD or IgE.

Thus the M components of both patients were related to the IgG Fc fragment and lacked L chains *Their* clinical picture also was compatible with IgG heavy-chain disease

Heavy chain disease may not be as rare a disease as thought before (5) Its diagnosis however is mainly based on immunoelectrophoresis and the use of more sophisticated immune reagents than those generally used in routine diagnostic work

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Acta path microbiol scandinav 75 353-362 1969

University Institute of Pathological Anatomy (Head G Teilum) and Department of Clinical Chemistry (Head P Astrup) Rigshospitalet University of Copenhagen Denmark

ACCELERATION OF SPONTANEOUS INTIMAL—SUBINTIMAL CHANGES IN RABBIT AORTA BY A PROLONGED MODERATE CARBON MONOXIDE EXPOSURE

By

J WANSTRUP K KJELDSEN and P ASTRUP

Received 9 viii 68

Hypoxia is believed to be a basic factor in atherogenesis (*Hueper* 1944) Recently *Astrup et al* 1967 demonstrated the enhancing effect of carbon monoxide on experimental cholesterol atheromatosis in rabbits

The aim of the present study is to evaluate the influence of a long lasting moderate carbon monoxide exposure on another atherosclerotic model *i e* the spontaneous degenerative and reparative vascular changes taking place in the intimal subintimal coats of the rabbit aorta

MATERIAL AND METHODS

The experiments were carried out on castrated male albino rabbits of Danish Country breed The animals were of pure breed and obtained from the same source The age at the start of the experiment was about 3 ½ months and the weights between 2700–3000 g The only source of feed was standard rabbit pellets and tap water *ad libitum*

Exposure to carbon monoxide ? airtight chambers (volume 1 m³) made of Perspex were used Each chamber contained individual cages for 5 animals The chambers were opened only once daily for feeding and rinsing purposes Carbon monoxide was mixed with atmospheric air as earlier described (*Astrup et al* 1967) Flow trough the chambers was maintained at 30 litres per minute

Methods for chemical determination Carboxyhaemoglobin was estimated spectrophotometrically as described earlier (*Astrup et al* 1967)

Experimental procedure After the usual acclimatization procedures (Astrup et al 1967) the animals were divided at random into 2 groups One group was exposed to carbon monoxide the other was used a control and kept in atmospheric air During the experimental period the exposure chambers contained 0 009 ± 0 004 per cent v/v carbon monoxide giving a mean carboxyhaemoglobin saturation of 11 per cent

After 3 months the animals were killed and macroscopic and microscopic autopsy performed The body weights were noted and the heart lungs and the aorta were removed in toto

Macroscopic examination The heart was excised opened and examined The weight after removal of clots was noted Specific attention was paid to the presence of focal myocardial changes

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Gratitude is expressed to *K Mellemgaard* MD and *J Georg* MD for performing weekly analyses (infrared spectrophotometry)

This work was supported by grants from *Foreningen til Hjertesygdommenes Bekæmpelse* and from *P Carl Petersens Fond*

The aorta was opened and the degree of localized intimal changes assessed and graded fro 0 to +++ as described earlier (Astrup et al 1967)

Microscopic examination According to routine sections from the heart aorta and lungs were taken for microscopy *Neutral buffered formalin fixation and conventional paraffin embedding was used The following staining techniques were used* Haematoxylin eosin van Gieson combined elastin and collagen staining (*Eskelund 1957*) staining for *fibrin like substan es (Lendrum et al 1962)* colloidal iron staining for acid mucinoid substances (*Mowry s modification*) and alizarin red staining for calcium Occasionally lipid staining was performed on cryostat sections

RESULTS

Macroscopic Findings

The CO exposed animals constantly showed increased amounts of serous fluid in the peritoneal pleural and pericardial cavities

Visible intimal changes were only seen in the *aortic arch* Occasionally small elevated plaques were found in both groups and macroscopically no significant differences between the two groups could be established on this basis (Table 1)

TABLE 1

Macroscopic and Microscopic Changes Graded from 0 to +++ of Rabbits on a Normal Diet Exposed or not Exposed to Carbon Monoxide for 3 Months

	Macroscopy		Aortae Microscopy	
Control group	Heart	Aortic arch plaque formation	Plaque formation	Focal intimal subintimal changes
64	0	0	0	0
66	0	+	++	0
62	0	0	0	0
1?3	0	0	+	0
65	0	+	++	0
111	0	+	++	0
63	0	0	+	0
117	+	++	+++	++
CO exposed group				
57	+	0	++	0
58	+	0	++	+++
51	0	+	+++	++
52	+	+	+++	++
53	+	+++	+++	++
55	0	0	+	+++
56	+	+	++	0
59	+	0	+++	+++
61	+	0	++	++

The mean weights of the *hearts* in the two groups showed no statistically *significant differences* (7 5 g ± 0 8 v 7 3 g ± 1 1) Most of the CO exposed animals (7/9) showed areas with hyperaemia and/or haemorrhages predominantly localized to the left heart and the

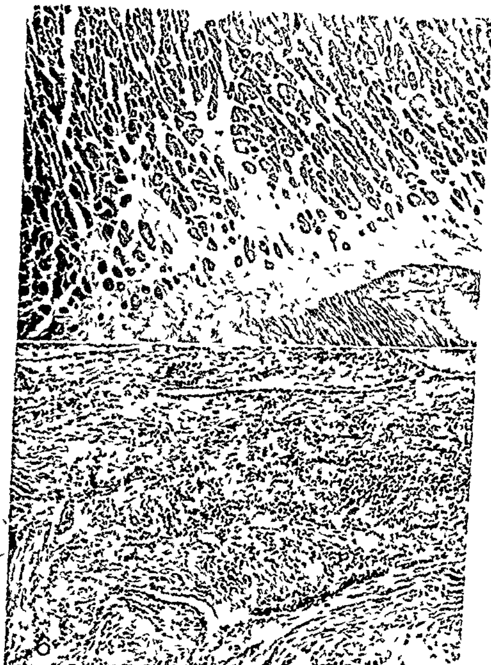




Figs 1–2

F g 1 A rta from CO exposed rabb t To the left slightly elevated plaque formation situated in intima subintima and inner media Prominent collagen formation is seen in addition to aggregation of elastic material in centre (black in picture) In a ljacent areas disturbance in intimal subint mal architecture oedema splitting of membranes and thickened intima (no nuclear staining) Combined ela tin-collagen stain (*Eskelund*) × 140

Fig Aorta from CO exposed rabbit In intima sul intima large oedematous "pool" are present Marked disarrangement of luminal elastic membranes is furthermore seen and endothelial cells ppear hypertrophied Vestopal embedded, 1 micron thick section Toluidine blue stain × 350



Figs 5–6

Fig 5 Myocardium from CO exposed rabbit Perivascular oedematous area with collagen proliferation extending int the myocardium causing varyin degrees of myofibrillar degeneration is seen Van Gieson stain × 140

Fig 6 From sub ndocardial oedematous area in CO exposed rabbit Prominent mononuclear cell infiltration and degeneration of myofibrils is seen Van Gieson stain × 140

of degeneration of the myocardial fibrils were also noted (Figs 5 6) Recent infarction was not found in the present study The coronary arteries showed no differences between the two groups

Other organs Some of the CO exposed animals showed small pneumonic foci and sometimes the pulmonary arteries exhibited marked intimal sclerosis eventually rising to fibrous plaque formation

DISCUSSION

Microscopy showed marked differences between the two groups in the present study concerning a spectrum of focal degenerative and reparative changes in the intimal subintimal coats No significant differences were observed in the degree of visible (macroscopic) intimal changes We feel justified in looking upon a number of the microscopic changes as changes pathogenetically related to atherosclerosis since mature atherosclerotic lesions (plaque formation) are usually accompanied by comparable marginally situated lesions Vascular changes characterized by disturbance in architecture splitting of membranes oedema and cellular proliferation are well described and also reported as spontaneous lesions in the rabbit aorta (*Haust & More* 1965) but oddly enough interpreted as Mönckeberg media type lesions They are also seen after severe acute carbon monoxide intoxications predominantly in medium and small sized vessels (*Rosenblath* 1925 *Petri* 1927 *Rix* 1932 *Yant et al* 1934 and *Pentschew* 1958)

The vascular changes described thus confirm the results obtained by earlier investigators that carbon monoxide exposure gives rise to endothelial hypertrophy and proliferation splitting up of the subintimal fibrillar texture and to a marked focal subintimal oedema Apparently attention has not earlier been paid to such changes in the aorta and especially not in connection with chronic CO exposure giving only 11 per cent carbon monoxide saturation of the haemoglobin The degenerative myocardial changes described in the present study are in accordance with earlier reports (*Campbell* 1929 *Ehrich et al* 1944 and *Lewey & Drabkin* 1944)

The presence of abnormal amounts of fluid in the serous cavities in the present experimental animals suggest an increased permeability of the endo and mesothelial membranes caused by carbon monoxide

The localized oedematous areas in the aorta comparable to the well known valvular and subendocardial oedema probably reflects a disturbance in the transport of fluid across the arterial wall An increased endothelial permeability for albumin is seen in human subjects after moderate carbon monoxide exposures (*Siggaard Andersen et al* 1968 and *Paull et al* 1968) suggesting that the oedema may originate in permeability changes The oedema could therefore be responsible both for the swelling and the splitting up of the vascular membranes and

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Acta path microbiol scandinav 75 363–366 1969

The Departments of Clinical Bacteriology (Head S Bergman) and Surgery (Head S Borgstrom) University of Umeå Sweden

LYMPHOCYTE RESPONSE TO PHYTOHAEMAGGLUTININ FOLLOWING CHOLECYSTECTOMY

By

S Bergman S Borgstrom and A Tarnvik

Received 15 viii 68

A reduced response of small peripheral lymphocytes to phytohaemagglutinin (P H A) has been found in many diseases with impaired immunological competence such as lymphoproliferative disorders (*Dameshek* 1967) ataxia telangiectasia (*Oppenheim et al* 1966) sarcoidosis (*Hirschhorn et al* 1964) and Whipple s disease (*Maxwell et al* 1968) and it has also been found after treatment with immunosuppressant drugs (*Hersh et al* 1967) Determination of the lymphocyte response to P H A has been suggested as a simple tool for assessing the immunological competence of peripheral lymphocytes (*Hersh et al* 1967 and *Dameshek* 1967) *Riddle & Berenbaum* (1967) and *Riddle* (1967) recently reported reduction of the lymphocyte response to P H A following surgery

In this study lymphocytes were obtained from patients before cholecystectomy and at various intervals after the operation The frequency of blastlike cells after culture of these lymphocytes in the presence of P H A was determined Serum samples from the same patients were tested on lymphocytes from a healthy person

MATERIALS AND METHODS

Eleven patients (10 ♀ 1 ♂) undergoing cholecystectomy were studied Two of the patients received blood transfusion 1 unit each the day after the operation There were no known co-existing diseases None of the patients received corticosteroids Neither the operation nor the later course was complicated. Blood samples were obtained before operation as well as about 24 hours and 5 to 29 days after the operation

Eight millilitre venous blood samples were collected into heparinized tubes containing 4 ml of 6 per cent dextran (Pharmacia) The tubes were placed at an angle of 4 for 1 hour in an ice bath The supernatant was then centrifuged 160 *g* for 5 min The cells were suspended in a concentration of 0 7 × 10^6 cells/ml in 8 tubes 1 5 ml in each Four tubes contained autologous serum obtained from the same sample as the lymphocytes the other contained pooled serum from healthy subjects The same pool was used throughout the investigation. The composition of the medium was

Parker TCM (with 125 units penicillin and 125 γ streptomycin/ml)	4 ml
serum	2 ml

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Parker TCM (with 125 units penicillin and 125 γ streptomycin/ml)	4 ml
serum	2 ml

Frequency of blastlike cells

32 28 24 20 16 12 8 4

-20 -13 -2 2 4 6 8 10 29

Days

A

Frequency of blastlike cells

40 36 32 28 24 20 16 12 8 4

-20 13 2 2 4 6 8 10 29

Days

B

Fig 1

Lymphocyte response to P H A before and after cholecystectomy *The arrows indicate the day of operation* The blood samples from that day were *all obtained before* operation A Autologous serum, obtained at the same time as the lymphocytes *present* in the medium B Pooled serum from healthy subjects present *in the medium*

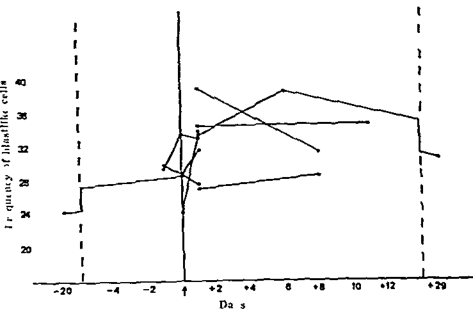


Fig 2

Effect of serum from cholecystectomy patients on the PHA response of lymphocytes from a healthy person. The arrow indicates the day of operation

P H A (45 mg/ml)	0 05 ml
Heparin (Vitrum 5 per cent aqueous solution without preservative)	0 05 ml

The P H A used was extracted from kidney beans (*Hall* 1964)

The cells were incubated at 37 C in 5 per cent CO_2—95 per cent air. After 70-72 hours the suspensions were centrifuged for 5 min (150 g) and the supernatants were pipetted off. The cells were suspended in 1 ml of 0 7 per cent Sodium citrate at 37 C for 15 min. The suspensions were centrifuged for 5 min (150 g) and the cells were fixed in 60 per cent acetic acid in 0 1 N hydrochloric acid for 5 min. The cells were stained in one drop of per cent orcein evenly distributed with a Pasteur pipette and then transferred to slide. Cover glasses were applied and fixed with honig cement. From each set of 4 tubes 800-1000 cells were examined. The frequency of large pale blastlike cells was determined. Mitoses were included but not medium sized and small cell forms

Samples of sera from the patients were stored at —70 C and later tested on lymphocytes from a healthy person

The results were examined statistically using the chi square test for heterogeneity

RESULTS

The frequency of blastlike cells was significantly lower ($p < 0.001$ in 6 patients and $0.001 < p < 0.01$ in 1 patient) in cultures of lymphocytes obtained about 24 hours after operation than in preoperative cultures. In this series of experiments (Fig 1a) the medium contained autologous serum. The results were essentially the same ($p<0.001$ in all 7 patients) when the medium contained pooled serum from healthy persons instead of autologous serum (Fig 1b)

The response to P H A usually began to recover within 10 days of the operation When cultured in autologous serum the cells from 6 out of 7 patients showed a significant increase in the blastoid frequency ($p<0\ 001$) When the cells were cultured in pooled homologous serum from healthy persons a significant increase from the 1st postoperative day was found in 6 patients ($p<0\ 001$)

Serum samples from 7 of the patients were tested on lymphocytes from a healthy person They did not affect the PHA response (Fig 2)

DISCUSSION

Riddle & Berenbaum (1967) and *Riddle* (1967) reported a reduced response to P H A of peripheral lymphocytes *in vitro* after various surgical operations This depression was seen also when autologous serum was not present in the medium Serum obtained from patients after operation did not affect lymphocytes from a healthy person The results of the present study agree with these observations The underlying mechanism of the reduced lymphocyte reactivity after surgery is obscure One obvious possibility is a hormonal effect on the lymphocytes

SUMMARY

The response of small peripheral lymphocytes to P H A was found to be reduced one day after cholecystectomy

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Acta path microbiol scandinav 75 367-369 1969

The Departments of Clinical Bacteriology (Head S Bergman) and Surgery (Head S Borgstrom) University of Umeå Sweden

EFFECTS OF SERUM FROM CHOLECYSTECTOMIZED PERSONS ON HELA CELLS

By

S Bergman S Borgstrom and A Tärnvik

Received 15 viii 68

The behaviour of HeLa cells in monolayer cultures is influenced by serum In the technique described by *Bergman* (1959) cells are allowed to attach to glass plates in the presence of various sera and other test solutions The cells are afterwards cultivated and finally counted Sera from patients with miscellaneous diseases have been tested by this technique (*Bergman* 1966)

In this study pre and postoperative sera from cholecystectomy patients were studied

MATERIALS AND METHODS

Serum samples from 10 female patients were studied None of the patients had received blood transfusions None had any known co existing disease Corticosteroids were not given Neither the operation nor the later course had been complicated

Venous blood samples obtained before and at various intervals after operation were centrifuged and the serum was immediately frozen at −70 C Serum samples from each patient were tested at the same time after storage at + 4 C for 7 days (*Bergman* 1966)

The experiments were performed on ordinary glass plates on which HeLa-cells 100 000 cells/ml were seeded in the serum to be tested After the cells had been attached to the glass for 5 hours they were allowed to grow for 18 hours and were then fixed stained and counted (*Bergman* 19 3) Twenty fields of vision per glass were examined under the immersion lens (plane 100/1 25 160/− ocular 12.5 ×) and 5 glasses were used for each test

RESULTS

Two preoperative serum samples from each of the 10 patients were tested (Fig 1) The average cell number was less than 2 in 2 (I S and H H) of the patients and between 4 and 16 in the remaining 8

After the operation the average cell number was below 2 on at least one occasion in all patients except J Ar In 7 cases (F D, S A J Al A H J H J K and F V) the average cell count fell significantly ($p < 0.001$) within about 24 hours to 4 days of the operation In serum samples from 5 of the patients the cell number began to increase within a month of operation In 2 cases (L D and A H) followed for 4 and 2 weeks the cell number showed no tendency to increase

The response to P.H.A. usually began to recover within 10 days of the operation. When cultured in autologous serum, the cells from 6 out of 7 patients showed a significant increase in the blastoid frequency ($p<0.001$). When the cells were cultured in pooled homologous serum from healthy persons, a significant increase from the 1st postoperative day was found in 6 patients ($p<0.001$).

Serum samples from 7 of the patients were tested on lymphocytes from a healthy person. They did not affect the PHA-response (Fig. 2).

DISCUSSION

Riddle & Berenbaum (1967) and *Riddle* (1967) reported a reduced response to P.H.A. of peripheral lymphocytes *in vitro* after various surgical operations. This depression was seen also when autologous serum was not present in the medium. Serum obtained from patients after operation did not affect lymphocytes from a healthy person. The results of the present study agree with these observations. The underlying mechanism of the reduced lymphocyte reactivity after surgery is obscure. One obvious possibility is a hormonal effect on the lymphocytes.

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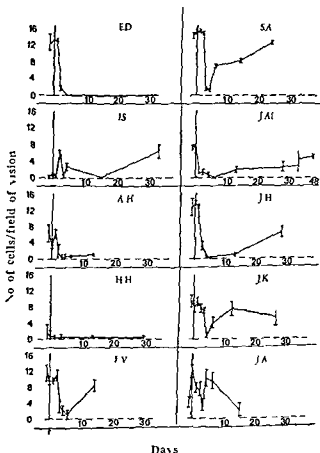


Fig 1

Effect of serum obtained before and after cholecystectomy on HeLa cells. The arrows indicate the day of operation. The blood samples obtained on that day were taken before operation. The standard deviation of each cell count is indicated by vertical lines.

Thus in 7 out of the 10 patients the relatively high preoperative cell numbers fell after the operation. In serum samples from 2 (I S and J Ar) of the patients the cell counts gave inconsistent results and in one (H H) the cell number was low throughout the investigation.

DISCUSSION

In a previous investigation (*Bergman* 1966) of normal sera with the present technique some of the samples had a cytotoxic effect on HeLa cells. Samples obtained on different occasions from a particular individual did not differ in their effect. In agreement herewith in preoperative tests in the present investigation the number of cells on the glasses varied from patient to patient. But in tests with postoperative sera the

They also noted that the slope of the first linear component was greater than that of the second indicating that the rate of increase in incidence was higher prior to age 40 than after age 40 *Lilienfeld & Johnson* concluded that *Clemmesen* s hook appeared to be a result of the change in the slope that occurred at 40 to 45 years of age

On the basis of these observations *Lilienfeld & Johnson* developed a hypothesis according to which the female population was assumed to consist of two subgroups one group was susceptible to the development of these tumours while the other was not susceptible They derived empirically a distribution which represented the proportion of females who remained susceptible at various ages By comparing this distribution with that representing the percentage of women still menstruating they noted a general similarity

The authors concluded that the hypothesis presented in their report was consistent with the idea that oestrogen and/or other hormones may form a necessary substance upon which other factors may act in order to produce the specific cancer

De Waard et al (1960 1964) divided their clinical breast cancer material according to conditions associated with adrenal oestrogen production and *concluded that there might be two types of breast* cancer One is largely limited to the reproductive years and ovarian oestrogens might play role in its genesis the second type is largely postmenopausal adrenal oestrogens being aetiologically involved

As pointed out breast cancer does not follow the general incidence pattern according to age In this paper it will be considered whether the age specific incidence curve for breast cancer supports the possibility that *Clemmesen* s hook is caused by two types or components of breast cancer

TABLE 1

Estimated Parameters of the Age Specific Incidence Curve for Carcinoma of the Breast in Nordic Countries

Country	Estimates of the parameters					
	a_1	t_1	σ_1	a_2	t_2	σ
Denmark	40	47 5	5 0	50	98 7	25
Finland	32	46 2	6 2	108	77 5	20
Norway	72	47 5	7 0	220	87 5	20
Sweden	57	47 5	6 0	240	8 5	20

Due to a recent proposal (*Hal ama*) the age specific incidence curve for cancerous diseases is assumed to have a normal distribution The incidence curve for breast cancer is resolved into two normal components applying the statistical methodology proposed by *Bhattacharya* (1967) This methodology requires no assumptions with regard to the aetiology of breast cancer only the existence of the components is considered

Acta path microbiol scandinav 75 370–374 1969

The Finnish Cancer Registry Helsinki Finland

THE PECULIAR AGE SPECIFIC INCIDENCE CURVE FOR CANCER OF THE BREAST—*CLEMMESEN'S* HOOK

By

MATTI HAKAMA

Received 23 viii 68

Breast cancer incidence as a function of age shows a continuous increase up to the age of forty to forty five years Then there occurs a leveling off or a slight diminution of the rates for the next few years of age after which the rates again show an increasing trend until very old age This pattern of age specific incidence rates is uncommon in tumours of other sites The diminution in breast cancer incidence rates at about the age of forty to forty five years was reported by *Clemmesen* in 1948 and is generally known as *Clemmesen's* hook (*Clemmesen* 1948 1965)

Several explanations for this leveling off have been given Since the period of diminution coincides with the average age at the onset of the menopause it has been assumed that the hormonal changes at that age decrease the risk of developing breast cancer

Clemmesen (1951) suggested that the hormonal unbalance caused by the subsequent disappearance of various hormones at this age may accelerate or postpone the development of an eventual mammary cancer thus transferring cases to other age classes and changing the shape of the curve

MacMahon (1957) advanced a hypothesis according to which the levelling off of the rates in data for a cross section of a population at a given time represented in reality an increased susceptibility to breast cancer among younger cohorts However as pointed out by *Haenszel* (1961) this hypothesis would imply an increasing trend in risk of developing mammary cancer an assumption which the available data does not support

According to *Lilienfeld & Johnson* (1955) the age specific incidence rates for breast cancer graphed on semilogarithmic paper could be resolved into two linear components one component that was present during the period from 20 to 40 years of life and a second that began at about 40 years of age and continued for the remainder of the life

Presented partly at the 15th meeting of the Nordic Cancer Union Oslo 1967

Breast cancer incidence in Denmark Finland Norway and Sweden is used The data are from the Finnish Cancer Registry and from the UICC publication Cancer Incidence in Five Continents (*Doll et al* 1966) The equation for two normal distribution curves added together is given by the equation

$$u(t) = a_1 \exp\left(-\frac{1}{2}\left(\frac{t-t_1}{\sigma_1}\right)^2\right) + a_2 \exp\left(-\frac{1}{2}\left(\frac{t-t_2}{\sigma_2}\right)^2\right)$$

The estimated parameters for this equation are given in Table 1 Figs 1 2 3 and 4 show the age specific incidence rates and the estimated incidence curves in the Nordic Countries

In the figures a logarithmic scale is used and the method of presentation corresponds to that of *Ellenfeld & Johnson* The curve consists of two additive normal components which resembles a combination of two straight lines Even if the curves fairly accurately fit the incidence rates the results cannot be accepted as a proof of two components of cancer of the breast This is because there is a large number of parameters six altogether which specify the curve and several other hypotheses of this complexity would provide a similar agreement with the observations However the agreement with the available data does not contradict the hypothesis according to which there are two components of breast cancer

Indirect support for the hypothesis of two forms of mammary cancer is provided by the fact that *Clemmesen*s hook is diminishing when mortality data instead of incidence data is considered and that the survival of young breast cancer patients is better than that to be expected on the basis of the biological behaviour of a malignant neoplasm by age compared with the prognosis of older patients Thus the prognosis of the first component might be better than the prognosis of the second component

There is some evidence in favour of the conclusion that *Clemmesen* s hook is caused by two components of a disease both of which are called breast cancer

SUMMARY

The age specific incidence rates from the Nordic Countries are employed to support the hypothesis according to which there may be two components of breast cancer the first with a peak at about 50 years of age and the second with a peak at considerably higher ages

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Acta path microbiol scandinav 75 375–398 1969

The Departments of Oral Pathology and Oral Surgery Royal Dental College
Aarhus Denmark

THE ADENOMATOID ODONTOGENIC TUMOUR

Ameloblastic Adenomatoid Tumour or Adeno Ameloblastoma

By

H P PHILIPSEN and H BIRN

Received 17 viii 68

The odontogenic jaw tumours i e tumours originating from tissue being or once having been engaged in odontogenesis cover a multitude of lesions from true neoplasms like ameloblastomas to hamartomatous lesions like the ameloblastic fibroma and odontoma The knowledge of these lesions has increased considerably within the last two decades An excellent review of odontogenic tumours in man and domesticated animals has been published by *Gorlin et al* (1961) This and more recent publications show that an increasing number of tumour variants and new entities are being recognized Among the less common odonto genic tumours is the so called ameloblastic adenomatoid tumour or adeno ameloblastoma The clinical and pathological features of this tumour seem fairly well established (*Gorlin et al* 1961 and *Bhaskar* 1964) An up to date review including cases not dealt with in the above mentioned reviews has however revealed additional features giving a more detailed picture of the tumour biology This report is an analy sis of the total material now available including three cases not pre viously published

TERMINOLOGY

The tumour in question has been reported in the literature under vary ing designations such as adenoameloblastoma (*Bernier & Tiecke* 1950 and 1956 *Thoma* 1955 *Topazian & Simon* 1960 *Link* 1963 *Bhaskar* 1964 a o) adenoameloblastic odontoma (*Bernier* 1955) epithelial tu mour associated with developmental cysts (*Stafne* 1948) tumour of enamel organ epithelium (*Lucas* 1957 *Shear* 1962) teratomatous odont oma (*Cahn* 1955) adenomatoid or pseudo adenomatous ameloblastoma (*Ishikawa & Mori* 1962) cystic complex composite odontoma (*Miles* 1951) drusiger Typ des Adamantinoms (*Langer* 1958) unusual pleo morphic adenoma like tumour (*Oehlers* 1956) and ameloblastic adeno matoid tumour or ameloblastic adenoma (*Gorlin et al* 1961 *Cina et al* 1961 and 1963 a o) In this paper the term *adenomatoid odontogenic tumour* (*A O T*) will be used for reasons later to be commented on

CLASSIFICATION

According to *Gorlin et al* (1961) the A O T can be classified as an epithelial odontogenic tumour *without* inductive changes in the connective tissue stroma In their classification which is a modification of the one suggested by *Pindborg & Clausen* (1958) the tumour is thus grouped together with the ameloblastoma and the calcifying epithelial odontogenic tumour (Table 1)

TABLE 1

Classification of Odontogenic Tumours

- Epithelial odontogenic tumours
  - No inductive changes in connective tissue
    - Ameloblastoma
    - Ameloblastic adenomatoid tumour (adeno ameloblastoma)
    - Calcifying epithelial odontogenic tumour
  - Inductive changes in connective tissue
    - Ameloblastic fibroma and ameloblastic sarcoma
    - Dentinoma
      - Immature (fibroameloblastic type)
      - Mature
    - Ameloblastic odontoma and ameloblastic odontosarcoma
    - Complex odontoma
    - Compound odontoma
- Mesodermal odontogenic tumours
  - Odontogenic myxoma and fibroma
  - Cementifying fibroma (periapical fibrous dysplasia?)
    - Periapical fibrous dysplasia type
    - Gigantiform type

From *Gorlin et al* 1961

CLINICAL FEATURES

It appears from the reviews by *Gorlin et al* (1961) and *Bhaskar* (1964) that the tumour is about twice as common in female as in male patients The tumour occurs at an early age usually in the second decade of life The maxilla is involved more frequently than the mandible according to Bhaskar showing a ratio of almost 2 1 The anterior region and in particular the cuspid region of the maxilla is the most common site of tumour development The tumour is frequently associated with an embedded tooth

Radiographically it appears as a radiolucent usually clearly demarcated area When associated with an embedded tooth the radiolucency surrounds the crown of the tooth giving the strong impression of a follicular (dentigerous) cyst

On close examination of the roentgenograms small irregular calcified bodies can sometimes be detected Apart from gradually increasing bony expansion there are few symptoms Failure of a tooth or teeth to erupt or persistence of a deciduous tooth or teeth are common changes causing patients to seek dental or medical advice

PATHOLOG

Macroscopically most tumours are rough
ing 1 to 3 cm in diameter The tumour h
sule The cut surface reveals one large or s [illegible]
of which may contain brownish or yellowish semisolid material In some cases the tumour appears solid Often one tooth or more rarely two are found embedded in the tumour mass or projecting into the cystic cavity

Microscopically the tumour tissue is composed of epithelial cells arranged either in sheets or in strands connected with one another forming a cribiform pattern between which is found a rather scanty connective tissue stroma containing thin walled vessels occasionally in considerable number Conspicuous within the cellular areas are structures of tubular appearance each consisting of a central space or lumen lined by a single layer of columnar epithelial cells resembling ameloblasts (*Miles* 1951 *Lucas* 1957 and *Oehlers* 1961) The lumen usually contains some homogenous eosinophilic material

In addition to forming tubular or ductlike structures the columnar cells also form convoluted bands or bodies arranged in complicated patterns Between the double row of opposed columnar cells making up the convoluted —sausageshaped— cords or bodies homogenous eosinophilic material can be found *Lucas* (1964) suggests that the tubule like structures are the results of a separation with tubule or duct formation of the opposed rows of cells in the sausage shaped bodies The areas intervening between the tubular structures and the sausage shaped cords or bodies are occupied by epithelial cells in some compressed and spindle shaped in other areas the cellular arrangement is quite loose and the cells assume stellate forms This epithelial stroma is continuous with the tubular structures and the convoluted bands or cords

Scattered throughout the tumour are small foci and infrequently larger masses of calcified bodies or globules *Oehlers* (1961) found rows of tall columnar epithelial cells in direct contiguity with some of the larger calcified deposits These cells resembled ameloblasts even more than did columnar cells found elsewhere in the tumour Oehlers suggests that these cells may be engaged in the production of the calcifying or calcified material which resembled enamel or enameloid tissue

Histochemical studies (*Miles* 1951 *Corlin & Chaudhry* 1958 *Shear* 1962 *Ishikawa & Mori* 19[illegible]) have revealed considerable controversy among the authors [illegible] true nature of the calcified material After having reviewed previous papers and observed staining reactions in two cases [illegible] A O T *Seuar & Duckworth* (1967 b) conclude that the calcified globules are epithelial [illegible] ducts and not dentine or predentine as suggested by *Shear* (1962) and that they probably resemble enamel in their composition

PATHOGENESIS

Several theories concerning the pathogenesis of the tumour have been put forward It has been suggested that the tumour is derived from salivary glandular tissue (*Oehlers* 1956) from ameloblasts prior to the stage of organization (*Lucas* 1957) from the enamel organ itself (*Gorlin & Chaudhry* 1958) and recently *Bhaskar* (1964) suggested that the tumour is in reality a follicular cyst with intracystic epithelial proliferation

Many authors regard the tumour as a variant of the ameloblastoma why they maintain the term adenoameloblastoma coined by *Bernier & Tiecke* 1950 An increasing number of investigators are inclined to deviate from this opinion as several features clearly separate the tumour in question from the true ameloblastoma

TREATMENT

It is generally agreed that enucleation is the treatment of choice During follow up periods running from eight years and a half to 36 years *Cina et al* (1961) observed no recurrence in five cases following conservative local treatment Lucas reported in 1964 no recurrence in his case previously reported (*Lucas* 1957) *Halperin et al* (1967) reviewed thirty five cases from the literature (not including the above mentioned reports by *Cina et al & Lucas*) and found no recurrence in any of the cases but as indicated by the authors only one case has been followed for more than 5 years the majority (27 out of 35 cases) having been followed from 6 months to 3 years

CASE REPORTS

Case 1

Referred to the Department of Oral Surgery Royal Dental College Aarhus complaining of a gradually increasing swelling of the anterior left side of the maxilla *The deformity had first been noticed two months previously The condition had* otherwise been asymptomatic Left upper first premolar had been extracted several years ago

Sex male

Age 27 years

Examination revealed a well defined bony expansion in the region of the left maxillary lateral incisor canine and first premolar *Slight displacement of the left* side of the upper lip and left ala of the nose Covering skin and oral mucous membrane was normal Left canine and first premolar were missing from the arch

Roentgenogram (Fig 1) showed an *embedded left permanent* canine Associated with it was a large cystic lesion resembling a follicular cyst The left lateral incisor and the second premolar both intact and vital showed deflection of their roots obviously caused by the expanding cystic lesion

Pre operative diagnosis cystis follicularis regio +3

Treatment under local anaesthesia a marsupialization was undertaken and the involved permanent canine was left *in situ* for the purpose of allowing it subsequently to erupt and the patient was referred to the Orthodontic Department for further treatment At operation the cyst lining the major part of which was left untouched *showed no suspicious features* as judged by examination through the

created window The temporary gauzepack inserted into the cavity was later substituted by an acrylic obturator

Histological findings the tissue removed at operation showed that it originated from an epithelium lined cavity no tumour tissue present The microscopy thus supported the clinical diagnosis

Postoperative course progress was uneventful and the involved canine was erupting toward the surface Six months later a tumourous thickening of the tissue surrounding the erupting canine was noted The planned orthodontic treatment was cancelled and the canine and adjacent tissue was removed in toto It shelled out easily Postoperative progress was uneventful To date (3 years later) there has been no evidence of recurrence (Fig 2)

Histological findings microscopy of the soft tissue surrounding the crown of the canine showed typical A O T (Figs 6 a and b)

Case 2[1]

The patient was referred to Dr *J Rud* Copenhagen for denudation of an embedded upper right canine and lateral incisor No symptoms present

Sex female

Age 16 years

Examination the permanent upper right canine and lateral incisor had not erupted The deciduous upper right canine was still present Otherwise no abnormalities could be found

Roentgenogram (Fig 3) revealed an embedded upper right permanent canine and lateral incisor Associated with the crown of the lateral incisor and extending distally towards the crown of the canine a well demarcated cystic lesion was found A pronounced root deflection was present at the apical one third of the lateral incisor

Pre operative diagnosis cystis follicularis ?+

Treatment with the intension of subsequent orthodontic treatment it was decided to do a marsupialization leaving the teeth *in situ* At operation under local anaesthesia the "cystic lesion" was found to be solid and tumour like The tumourous tissue shelled out easily leaving a smooth walled bone cavity The temporary gauzepack was later substituted by an acrylic obturator

Histological findings (Prof *J J Pindborg*) microscopy showed A O T (Figs 7 a and b)

Postoperative course healing progressed uneventfully and the patient received orthodontic treatment after eruption of the involved teeth When seen 3 years later the lateral incisor had reached its normal position in the arch while the canine still needed orthodontic treatment (Fig 4) There was no evidence of tumour recurrence

Case 3

Admitted to Department of Oral Surgery School of Dentistry University of Minnesota Minneapolis complaining of a slow growing swelling involving the left maxillary canine premolar area The condition had otherwise been asymptomatic

Sex male

Age 19 years

Examination revealed a bony expansion of the left maxillary canine premolar region The left canine was missing from the arch All teeth present in the region were vital

Roentgenogram (Fig 5) revealed an embedded permanent left upper canine Around and posterior to the crown and root was a 3 by 3 cm cystic lesion containing numerous minute areas of calcification

Pre operative diagnosis calcifying tumour of the left maxillary canine premolar region

[1] The author wishes to thank Dr *J Rud* Copenhagen and Prof *J J Pindborg* Head of Department of Oral Pathology Royal Dental College Copenhagen for permission to include the case in this article

[2] The authors are indebted to Dr *R A Vickers* Dept. of Oral Pathology University of Minnesota Minneapolis USA for permission to include ... article

PATHOGENESIS

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marized in Table 2 seem to present sufficient evidence according to the description given earlier in the present paper to be diagnosed as A O T

As shown in Table 2 a number of cases have been published twice a fact not always considered in previous reviews

RESULTS

In the previously mentioned classification the adenomatoid odontogenic tumour is grouped together with the ameloblastoma and the calcifying epithelial odontogenic tumour (Table 1) It was considered of interest to analyse the relationship between these three tumour entities and available data are summarized in Tables 3 and 4 and further treated in Figs 9 and 10

The A O T occurs (or is recognized) predominantly in the second decade of life Fig 9 shows a distinct peak in this age group Almost three out of every four cases are found in patients between the age of 10 and 19 years Thus the A O T is clearly characterized in that it is a tumour of young age An analysis of the age distribution *within the second decade group* reveals a maximum occurrence (44 out of 52 cases) in patients of sixteen plus/minus three years of age It seems therefore that the tumour can be further characterized as a *tumour of the teen-age*

On the other hand the ameloblastoma is found in the third and fourth decade of life and compared with the A O T there is less marked difference in incidence between the age groups

In spite of the rather restricted number of published cases our knowledge concerning the calcifying epithelial odontogenic tumour seems to indicate a tumour of the fourth and fifth decade of life

Table 4 and Fig 10 show that the adenomatoid odontogenic tumour is more common in the maxilla than in the mandible The reverse seems to be true for the ameloblastoma and the calcifying epithelial odontogenic tumour

As to sex distribution the A O T shows a slight predominance in *female* patients The calcifying epithelial odontogenic tumour shows however in *male* patients a predominance of almost the same strength as the A O T shows female dominance whereas the ameloblastoma shows an almost 1 1 ratio

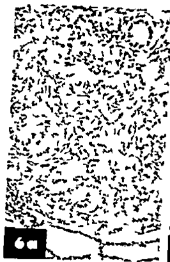
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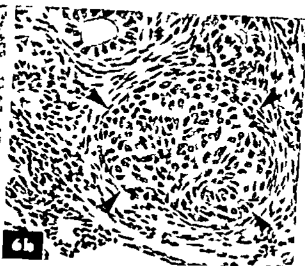
Figs 6 8

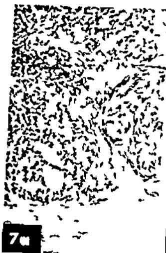
Fig 6 a and b Low and high power pl t m cr graphs showing typical tum u t ue with characteristic du t l ke tructure and convoluted bod e (a r v) (present case 1)

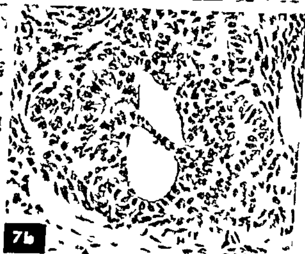
Fig 7 a and b Low and high power p h t micrographs showing tumour t ssue with ductlike structures (pre t t case 2)

Fig 8 a and b Low and high power photomi rographs showing tumour tissue and several areas of calcification (present case 3)


6a


6b


7a


7b


8a


8b

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Figs 6–8

Fig 6 a and b Low and high power photomicrographs showing typical tumour tissue with characteristic ductlike structure and convoluted bodies (arrows) (present case 1)

Fig 7 a and b Low and high power photomicrographs showing tumour tissue with ductlike structures (present case 2)

Fig 8 a and b Low and high power photomicrographs showing tumour tissue and several areas of calcification (present case 3)

TABLE

Adenomatoid Odontogenic

Review of

Case No	Author	Year of publ	Sex	Age yrs	Location
1	*Ghosh*	1934	♂	18	maxilla
2	*Darlington & Lefkowitz*	1936	?	?	mandible
3	*Figi & Stafne*	1938	♀	16	maxilla
4	*Stafne*	1948	♂	15	maxilla
(3)			♀	16	maxilla
5			♀	14	maxilla
6	*Bernier & Tiecke*	1950	♂	13	mandible
7	*Miles*	1951	♂	18	mandible
8	*Thoma*	1955	♀	11	mandible
(6)	*Bernier & Tiecke*	1956	♂	13	mandible
9			♀	13	maxilla
10			♀	26	mandible
11			♂	14	mandible
12			♂	17	maxilla
13			♂	13	mandible
14			♀	21	maxilla
(8)			♀	11	mandible
15			♀	20	mandible
16	*Oehlers*	1956	♀	15	maxilla
17	*Ishikawa*	1957	?	?	maxilla
18	*Lucas*	1957	♀	19	maxilla
19	*Belloso et al*	1958	♀	16	maxilla
20	*Gorlin & Chaudhry*	1958	♀	18	maxilla
21			♀	13	maxilla
22			♀	16	maxilla
23	*Ono*	1959	♀	17	mandible
24	*Topazian & Simon*	1960	♀	10	maxilla
25			♀	12	maxilla
26			♀	25	maxilla
27			♀	15	maxilla
28	*Gorlin et al*	1961	♀	5	maxilla
29			♂	6	mandible
30			♀	11	maxilla
31			♀	25	mandible
(20)			♀	18	
(21)			♀	13	
(22)			♀	16	

2
Tumour
literature

Tentative clinical diagnosis	Impacted tooth	Present author's comment
dentigerous cyst (d c)	+8	
d c	4—	
l c	3+	
d c d c globulo maxillary cyst	4+ 3+ no	identical with case reported by *Figi & Stafne* 1938
d.c	1—	
d c	—8	
radicular cyst/ameloblastoma	no	
d.c ? ? d c ? d c d c radicular cyst/ameloblastoma ?	1— ? no —3 +3 or 3+ 3— 4+ no no	identical with case reported by the authors in 1950 identical with case reported by *Thoma* 1953
d c	3+	
l c	yes	further details see *Ishikawa & Mori* 1962
?†	n	
?	?	
? d c ?	no +3 extraosseous	
d c	?	
d c d c d c d c	+3 +3 3+ +3	
	? ? ? ?	identical with case reported by *Gorlin & Chaudhry* 1958

TABLE

Case No	Author	Year of publ	Sex	Age yrs	Location
32	*Oehlers*	1961	♂	12	mandible
33			♂	20	maxilla
34	*Tiecke & Shira*	1961	♀	34	maxilla
(17)	*Ishikawa & Mori*	1962			
35			♀	13	maxilla
36			♀	16	maxilla
37			♂	30	mandible
			♀	18	mandible
38	*Shear*	1962	♂	25	mandible
39			♀	15	maxilla
40	*Cina et al*	1963	♂	27	mandible§
41			♀	16	mandible
42			♂	31	mandible
43			♂	11	mandible
44	*Lint*	1963	♂	19	maxilla
45	*Monteleone & Giordano*	1963	♀	15	mandible
46	*Root*	1963	♀	15	maxilla
47	*Rosenberg & Cru*	1963	♂	18	mandible
48			♀	[illegible]	maxilla
49	*Shira & Bhaskar*	1963	♂	25	maxilla
50	*Bhaskar*	1964	♀	9	
51			♀	[illegible]	
52			♀	13	
53			♀	15	
54			♀	16	maxilla
55			♀	16	11 cases
56			♀	[illegible]	
57			♂	17	mandible
58			♂	18	4 cases
59			♂	18	
60			♂	[illegible]	
61			♂	23	
(49)			♂	[illegible]	
62			♂	[illegible]	
63			♂	38	
64	*Das et al*	1964	♂	19	mandible
65	Persson	1964	♂	14	mandible
66	*Walker & Pfafflin*	1964	♂	15	maxilla
67	*Maranda*	1965	♂	13	maxilla
68	*Hornova*	1965	♀	[illegible]	maxilla
69	*Lentrodt & Shimi u*	1965	♀	15	maxilla

2 (cont)

Tentative clinical diagnosis	Impacted tooth	Present author s comment
d.c d c	—3 1+	
?	no	
d c ? d c ?	2+ ? 3— ?	identical with case reported by *Ishikawa* 1957
d c d c	? +2	
? d c ? ?	? 1— or 2— ? no	
d c	3+	
d c	3—	
d c	+3	
?	no no	
dont tum ur?	no	
	9 ca es were associated with impacted teeth	
		possibly identi al with case re ported by *Shi a & Bhaskar* 1963
d	– 3	
t	+—	
d	+ ?	
i	+3	
i	+3	
i	3+	

TABLE

Case No	Author	Year of publ	Sex	Age yrs	Location
32	*Oehlers*	1961	♂	12	mandible
33			♂	20	maxilla
34	*Tiecke & Shira*	1961	♀	34	maxilla
(17)	*Ishikawa & Mori*	1962	♀	13	maxilla
35			♀	16	maxilla
36			♂	30	mandible
37			♀	18	mandible
38	*Shear*	1962	♂	25	mandible
39			♀	15	maxilla
40	*Cina et al*	1963	♂	27	mandible§
41			♀	16	mandible
42			♂	31	mandible
43			♂	11	mandible
44	*Link*	1963	♂	19	maxilla
45	*Monteléone & Giordano*	1963	♀	15	mandible
46	*Root*	1963	♀	15	maxilla
47	*Rosenberg & Cru*	1963	♂	18	mandible
48			♀	10	maxilla
49	*Shira & Bhaskar*	1963	♂	25	maxilla
50	*Bhaskar*	1964	♀	9	
51			♀	12	
52			♀	13	
53			♀	15	
54			♀	16	maxilla
55			♀	16	11 cases
56			♀	17	
57			♂	17	mandible
58			♂	18	4 cases
59			♂	19	
60			♂	19	
61			♂	23	
(49)			♂	25	
62			♂	32	
63			♂	38	
64	*Das et al*	1964	♂	19	mandible
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67	*Maranda*	1965	♂	13	maxilla
68	*Hornova*	1965	♀	24	maxilla
69	*Lentrodt & Shimizu*	1965	♀	15	maxilla

? (cont)

Tentative clinical diagnosis	Impacted tooth	Present author s comment
d c d c	—3 1+	
?	no	
d c ? d c ?	2+ ? 3— ?	identical with case reported by *Ishikawa* 1957
d c d c	? +2	
? d c ? ?	? 1— or 2— ? no	
d c	3+	
d c	3—	
d c	+3	
?	no no	
odont tumour ?	no	

9 ases we e associated with impacted teeth

possibly identical with case reported by *Shira & Bhaskar* 1963

d c	– 3	
d c	4—	
d	+2	
l c	+3	
l c	+3	
d c	3+	

?

TABLE

Case No	Author	Year of publ	Sex	Age yrs	Location
70	*Spouge*	1967	♂	12	maxilla
71	*Seward & Duckworth*	1967a	♂	16	maxilla
(7) 7?	*Halperin et al*	1967	♂ ♀	18 17	mandible maxilla
73	*Mallon et al*	1968	♂	16	mandible
74 75 76	Present cases	1968	♂ ♀ ♂	27 16 19	maxilla maxilla maxilla

anterior region i e within the incisor canine and first premolar region
§ posterior region i e within the second premolar and molar region

TABLE

Distribution of Tumou

Age group Tumour	0–9 No	0–9 %	10–19 No	10–19 %	20–29 No	20–29 %	30–39 No	30–39 %	40–49 No	40–49 %
Adenom odont tumour	3	4	54	72	12	16	5	7	1	1
Ameloblastoma	24	4	113	19	172	28	127	20	81	13
Calc epith odont tumour	0	0	1	3	5	17	6	21	7	24

The figures concerning the A O T are based on all cases shown in Table 2 except case no 2

TABLE

Distribution of Tumour Cases

Tumour	Location Max No	Location Max %	Location Mand No	Location Mand %	Total No	Total %
Adenom odont tumour	48	63	28	37	76	100
Ameloblastoma	173	19	752	81	9 5	100
Calc epith. odont tumour	10	34	19	66	29	100

The figures concerning tumour location *for the A O T are based* on all cases shown in T ble 2

The figures concerning sex distribution for the A O T are based on all cases *shown in* Table 2 except case no 2

2 (cont)

Tentative clinical diagnosis	Impacted tooth	Present author s comment
d c	3+ or +3	
d c	4+	
d.c d c	—8 +2	identical with case reported by *Miles* 1951
d c	3—	
d c d c calcifying tumour	+3 2+ +3	

† when re examined in 1964 (*Lucas* 1964) thought to be a dentigerous cyst
? no information available

3
Cases According to Age

50–59		60–69		70–79		Total		Author
No	%	No	%	No	%	No	%	
0	0	0	0	0	0	75	100	*Philipsen & Birn* 1968
50	8	39	6	12	?	621	100	*Small & Waldron* 1955
6	21	3	11	1	3	29	100	*Potoliege et al* 1965 *Pindborg* 1966 *Decker & Laffitte* 1967 and *Abrams & Howell* 1967

Only case 4 is included as cases 1 2 and 3 are dealt with in *Pindborg* s paper *(1966)*

4
According to Locati n and Sex

Sex						
♀		♂		Total		Author
No	%	No	%	No	%	
40	53	35	47	75	100	*Philipsen & Birn* 1968
473	48	514	5?	987	100	*Small & Waldron* 1955
12	4	17	58	29	100	*Poti liege et al* 1965 *Pindborg* 1966 *Decker & Laffitte* 1967 and *Abrams & Howell* 1967

Only ca e 4 is included as cases 1 2 and 3 are dealt with in *Pindborg* s paper *(1966)*

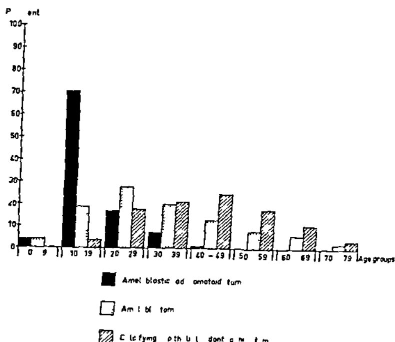


Fig 9
Age distribution of tumour cases based on figures from Table 1

Fig 11 shows published cases (extracted from Table 2) according to location It is evident that the anterior region of the maxilla is the site of predilection Among the total number of cases of which sufficient information was available (51) 48 were located in the anterior jaw regions Out of these 48 cases 36 (75 per cent) were associated with embedded teeth and 37 were primarily diagnosed as follicular (dentigerous) cysts Two of the three cases found in the posterior region of the maxilla and mandible were clinically diagnosed as follicular cysts

One may conclude that the majority of tumours (A O T) are associated with impacted teeth and most often primarily misdiagnosed as follicular (dentigerous) cysts

In a number of reports (but unfortunately by far not in all) the particular embedded tooth (or teeth) involved in the tumour lesion is stated Fig 12 shows that the cuspid region of the maxilla is by far the most common site of tumour development

DISCUSSION

The present review based on 76 cases of adenomatoid odontogenic tumours clearly shows its unique position within the group of odontogenic tumours

Although described by *Ghosh* (1934) and *Darlington & Jefkowitz* (1936) the tumour was first recognized as an entity by Stafne in 1948

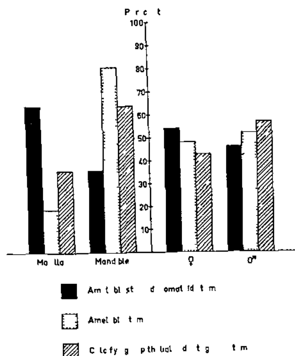


Fig 10

Distribution of tumour cases according to location (left) and sex (right) based on figures from Table 4

who used the term epithelial tumour associated with developmental cysts of the maxilla *Lucas* (1957 and 1964) among several authors gives credit to *Dreybladt* (1907) for being the first to describe this tumour A detailed study of Dreybladt s non illustrated thesis and a review of the articles to which he refers leaves great doubt as to the identity between his Pseudo ad noma Adamantinum and the tumour in question For this reason his case is not included in the present report

As to *age distribution* the r ults ar in agreement with earlier reviews (*Gorlin et al* 1961 ir *Phi ter* 1964) The increased number of cases now available f an d s t nd furthermore to strengthen the concept In addition r row t f h a e range within which it occurs has been demon tr d a *j ority of patients are in their teens* On this point the A O can arly separated from the ameloblastoma and the calcifyi itr dontogenic tumour the latter two tumours most often occ r ing in the third to fifth decade of life

Gorlin et al (1961) and *I i kar* (1964) found a maxillary pre dominance with regard to *site of tumour formation* The present review supports their results and shows that almost two thirds of the reported cases are located in the maxilla Again there is a

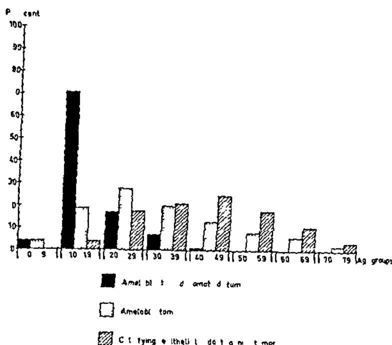


Fig 9
Age distribution of tumour-cases based on figures from Table 3

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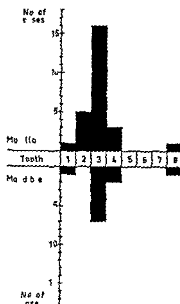


Fig 12
Distribution of tumour cases according to embedded tooth involved Based on cases nos 1–4 6 7 11 14 16 17 [illegible] [illegible] 27 [illegible] 33 [illegible] [illegible] 44–46 [illegible] 76 (*Table 2*)

If one compares the figures *from recent reviews with data now* available (Table 4) there is an obvious equalization of the *sex ratio and it* seems no longer justified to maintain the statement that the A O T is about twice as common in female patients (*Gorlin et al* 1961 *Shafer et al* 1963 and *Lucas* 1964 a o) Comparing the three tumours there is probably no significant difference in sex distribution although it is evident that *more material larger series* of the adenomatoid odontogenic tumour and the *calcifying epithelial odontogenic* tumour are needed to clarify the question

The A O T is characterized in that it is *associated with embedded teeth* Almost 75 per cent of the reported cases were found to be associated with embedded teeth *Khaskar* (1964) found 26 out of 41 cases (or 63 per cent) The present review emphasizes the strong relationship between tumour and embedded teeth and thus strengthens the concept of an odontogenic origin of the A O T

From a clinical point of view it is of importance *to notice the frequency* with this tumour is [illegible] *as a follicular (dentigerous) cyst* Three out of every 4 tumour cases have been diagnosed primarily as a *follicular* cyst The findings [illegible] or more definitely the result of microscopy proved the existence of tumour tissue rather than follicular cyst At this point we would like *to* stress the importance of a thorough microscopic examination of all cystic jaw lesions

Pindborg (1966) states that among the [illegible] cases of calcifying epithe

lial odontogenic tumours of which information were available as to the presence of embedded teeth 13 (87 per cent) had a non erupted tooth associated with the tumour In a study of 88 cases of ameloblastoma *Kane* (1951) found 29 cases (33 per cent) where an association existed *between the tumour and follicular* (dentigerous) cyst It seems characteristic of epithelial odontogenic tumours with no inductive changes in the connective tissue (Table 1) *to have a more* (A O T and calcifying epithelial odontogenic tumour) or less (ameloblastoma) pronounced association with embedded teeth or *follicular cyst surrounding such* teeth

A more detailed analysis of the exact *location* of the A O T *within the jaws* showed a strong predilection for the maxillary cuspid region Sixteen out of 38 cases developed in association with an embedded upper permanent cuspid

It is interesting and probably of significance to recall that in the maxilla the permanent cuspid is the tooth most *frequently* involved in follicular (dentigerous) cyst formation (*Bernick* 1949) The association between dentigerous cysts and the A O T development which *Bhaskar* (1964) believes is definite (the tumour is in a reality a follicular *cyst the lining of which proliferates into the lumen of the cystic* cavity) is then made quite reasonable On the other hand it is worth pointing out that the maxillary cuspid is considered the tooth most frequently embedded excluding the third molars (*Vergopoulos* 1958) The tumour pathogenesis is therefore not necessarily linked to the concept of follicular cyst derivation but might as well be assumed to be a development from the reduced enamel epithelium surrounding the crown of the embedded tooth as suggested by *Lucas* (1957)

In reviewing the reported cases of adenomatoid odontogenic tumour where an association with embedded teeth is evident no abnormalities or hypoplastic defects have been noticed in the crown of the embedded tooth This seems to support the concept *that the tumour is developed* either from the reduced enamel epithelium surrounding the crown or *from the epithelium lining the cystic cavity* One *would expect* a faulty odontogenesis if the tumour developed from the enamel organ epithelium at a stage where histo and morphodifferentiation *had not been* completed Follicular (dentigerous) cysts usually develop after formation of the crown of the tooth thus leaving no traces of defects qualitatively or quantitatively in the enamel

In one of the cases presented here (case no 74 Table 2) a pronounced vascularity was found in the stroma We *consider it to be a* secondary phenomenon and there is no indication of the vascular proliferation being neoplastic *Lucas* (1957) has rightly pointed out that a number of previously published cases of haemangioameloblastoma probably are *examples of A O T* with a marked vascular stroma *It is* therefore of great importance not to be misled by a pronounced vascularity in the adenomatoid odontogenic tumour and diagnose *the lesion*

as a vascular ameloblastoma or haemangioameloblastoma like the case recently reported by *Shklar & Cataldo* (1965). A study of their photo micrographs and description clearly shows a vascular A O T occurring in a thirteen year old boy. The tumour was located between the right mandibular cuspid and first premolar. In spite of these clinical features typical of the A O T the diagnosis vascular ameloblastoma was maintained and the thirteen year old boy was treated by surgical excision with sacrifice of adjacent teeth for a wide margin.

Ductlike structures lined by columnar epithelial cells and areas showing various stages of attempted duct formation are histomorphological features typical of the A O T. Most investigators agree that the tumour is not of true glandular origin. The use of the prefix adeno— might be misleading if not properly interpreted whereas the term adenomatoid conveniently covers the characteristic gland *like* structures often found.

The columnar epithelial cells may have a superficial resemblance to ameloblasts but to us no convincing evidence has been presented indicating true ameloblastic properties (i e enamel formation). In the case reports presented by the present authors calcified or semicalcified structures were found in varying amounts but no indication of enamel or dentine like structures could be demonstrated.

If true dental hard tissues do occur in these tumours as suggested by *Oehlers* 1961, *Shear* 1962, *Ishikawa & Mori* 1962, *Seward & Duckworth* 1967 b a o this means that the tumour possesses inductive abilities. As a consequence the tumour cannot rightly be classified as an epithelial odontogenic tumour *without* inductive changes using the classification by *Gorlin et al* (1961) shown in Table 1.

Gorlin & Chaudhry (1958) point to the similarity to ameloblastic fibromas and ameloblastic odontomas with regard to clinical behaviour and history. Concerning the histological picture however there are very few if any similarities between the A O T and these tumours.

There seems to be no justification for maintaining the term adeno ameloblastoma or for that matter any term using the suffix ameloblastoma for the tumour in question. On the basis of the present knowledge it is fully justified to consider the adenomatoid odontogenic tumour a separate tumour entity. To classify it as a subtype of the "simple or true" ameloblastoma and maintaining *ameloblastoma* in the terminology is misleading and might prove disastrous (*Dodge* 1965). The surgeon may perform radical resection which is the accepted and adequate treatment in case of true ameloblastoma but based on present knowledge a tragic error in the case of an adenomatoid odontogenic tumour. We therefore strongly suggest a rapid change in terminology.

To us the most appropriate designation and one which matches the term calcifying epithelial odontogenic tumour (Table 1) would be *adenomatoid odontogenic tumour* as proposed in this paper. This non committal term avoids ameloblastoma or ameloblastic. It stresses

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Acta path microbiol scandinav 75 399-414 1969

Institute of Pathology II (Head Prof S Falkmer) University of Umeå
Umeå Sweden

PANCREATIC ISLET MORPHOLOGY IN DIABETIC CHINESE HAMSTERS

A Light and Electron Microscopic Study

By

LENNART BOQUIST

Received 20 viii 68

Several strains of rodents are nowadays known in which spontaneous diabetes occurs apparently of pancreatic origin (*cf Renold & Dulin* 1967) One of these is the Chinese hamster in which the disease in many respects is similar to human diabetes mellitus (*Meier & Yerganian* 1959 *Gerritsen & Dulin* 1967 *Gundersen et al* 1967)

The present report is part of an attempt to perform a systematic study of the endocrine pancreas of the Chinese hamster in normal (*Boquist* 1967 a b and c) and various experimental and pathological states Previously the histophysiology including the regenerative phenomena has been studied in alloxan diabetes (*Boquist* 1968 a and b) and in dietary zinc deficiency (*Boquist* 1967 d) Some light microscopic (*Meier & Yerganian* 1959 *Carpenter et al* 1967 *Malaisse et al* 1967) and preliminary ultrastructural (*Luse et al* 1967) studies of the islet tissue mainly of diabetic Chinese hamsters have also been reported Against this background it was thought worth while to report our findings up to now concerning the qualitative pancreatic islet morphology in spontaneous diabetes in this animal

MATERIAL AND METHODS

The animals were selected from two strains of the Chinese hamster both originating from Dr Yerganian's colony in Boston and systematically inbred by brother sister mating first in Boston and then at the Charles H Best Institute in Toronto and the Institute of Genetics in Lund respectively and lastly at the Institute of Pathology in Umeå since 1964 From these strains 17 hamsters of both sexes exhibiting diabetic symptoms (*Meier & Yerganian* 1959) were selected for the present study These animals did not receive any therapy with insulin or any other antidiabetic drug By means of previously described procedures (*Boquist* 1967 c) it was noted that the animals showed persistent glucosuria and hyperglycaemia Some data concerning the diabetic hamsters are given in Table 1 The designation of the animals mentioned below refers to this table The hamsters were fed conventional pellets for laboratory rodents *ad libitum* and had free access to drinking water At predeter

This work was supported by grants from the *Swedish Medical Research Council* (Project No h67 12X 718 02 and h68 12X 718 03) and from the *Medical Faculty of the University of Umeå*

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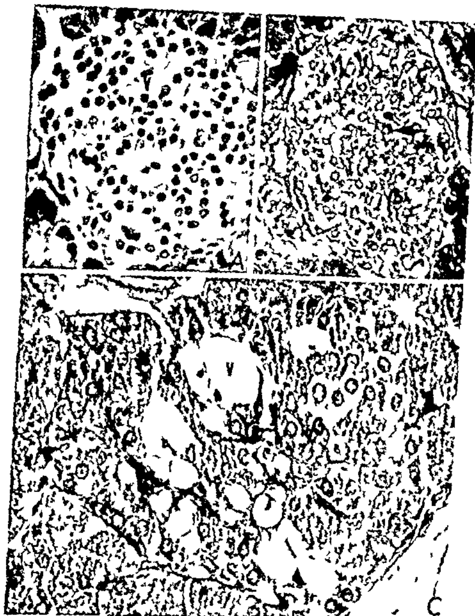


Fig 1

(A) Islet showing nuclear polymorphism and pyknosis among the β-cells that occupy most of the islet Some of these cells contain cytoplasmic vacuoles (v) (Animal No 3) Bouin's fixative van Gieson's stain × 250

(B) Islet showing a few granulated β cells (arrow) Most of the central cells that are slightly stained or unstained are probably β cells (β) with decreased granulation (Animal No 13) Bouin's fixative Chrome alum haematoxylin stain × 250

(C) Islet demonstrating large mainly intercellularly localized vacuoles (v) Most of the cells in the central part are probably β-cells (β) with decreased granulation (Animal No 15) Bouin's fixative Aldehyde fuchsin stain × 600



Fig ~

Low po r lectr n micrograph f i let sho ing β cells (β) with sp rse granulation *The Golgi c mplex (G) is rather prominent in some of the β cells Cells interpreted* as α_1- (α_1) α - (α) an l agranular (γ) cell are also seen (Animal N 10) × 6 000

Capti n t F gur s 1–11

All light n l ele tron micr graph c m fr m pancreati islet tissue of d abetic Chin se ham ters (Th ani n l numbers refer to Table 1)

6 CT TH 7 J

mined intervals the animals were sacrificed and specimens were taken from the pancreas for light and electron microscopic examination The morphology of the vessels was not included in the present report as it will be the subject of a future study The details concerning the light and electron microscopic procedures were given in preceding studies (Boquist 1967 a and b) In addition pancreatic specimens were fixed in alcoholic formalin and the sections were stained with the periodic acid Schiff reagent (PAS) with and without prior digestion with diastase for light microscopic detection of glycogen (*Lillie* 1965) For the demon tration of amyloid the staining method of *Puchtler et al* (1962) was applied and the sections were examined in polarized light (*Missmahl* 19,7) The occurrence of fat was studied by the procedure of *Chiffelle & Putt* (1951)

RESULTS

Light Microscopic Findings

General appearance There were no signs of hyalinization amyloid deposition fibrosis bleeding or fatty infiltration Only in one animal (No 2) slight infiltration of lymphocytes and plasma cells were ob served around some of the islets No alterations of nerve structures were recorded

β cells These cells occasionally showed nuclear polymorphism and pyknosis (Fig 1 A) Differential granule staining procedures often revealed β cells with sparse granulation and cells devoid of granules (Fig 1 B and C) The cytoplasm was frequently vacuolized and large vacuoles were also localized intercellularly (Fig 1 C) The vacuolized cells contained PAS positive material that was abolished by diastase digestion and was interpreted as glycogen Infrequently there was necrosis of central cells probably β cells with some remaining cellular *debris*

Other pancreatic parenchymal cells The α_1 and α_2 cells were un affected Some of the cells that were negative in granule stained sections were probably of agranular type There were no changes in the ductal ductular or acinar cells

Ultrastructural Findings

β cells In these cells granulation was frequently decreased (Figs 2 and 3) The mitochondria sometimes showed swelling and disintegra tion Some cells particularly those with decreased granulation ex hibited well developed endoplasmic reticulum and prominent Golgi complex Rather often the cisternae of endoplasmic reticulum were dilated and on occasion they cont uned amorphous material (Fig 4) App irent cytoplasmic glycogen deposits of varying size were often pres nt (Fig 5) In cells with glycogen infiltrates unaffected cyto plasmic organelles occurred Though the amount of secretory granules was decreased *in many of these* cells there was no clear association be tween glycogen deposition and decreased granulation Accumulations of electron lucent material were also found (Fig 4) Unaffected cellular organelles could be seen also in cells with electron lucent material There were no changes in the cell membranes Cilia with normal

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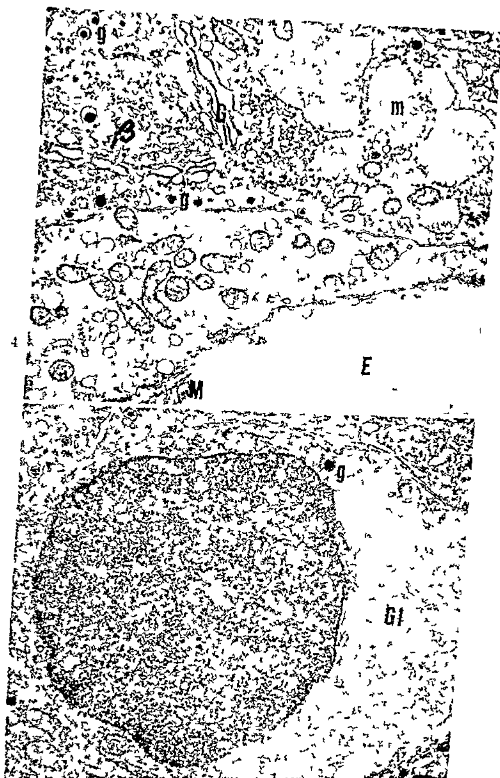
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g
G
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structure were found in some cells (Fig 6) Cells with electron dense cytoplasm and secretory granules seemingly of β type occasionally occurred (Fig 7)

Cytoplasmic bodies of varying appearance and size were frequently found Some of these were myelin like (Figs 8 and 9) and others showed similarities with so called autophagic vacuoles Some were seemingly formed by two three or more aggregated secretory granules (Fig 9) and some were rather large and dense (Fig 10) These cyto plasmic bodies were usually surrounded by membranes and had elec tron density similar to that of secretory granules Cytoplasmic bodies were also seen to consist of membrane enclosed particles with appear ance and electron density reminiscent of secretory granules (Fig 11)

On occasion necrotic changes occurred in the β cells

Other cellular elements The α cells were not changed The α_1 cells were often unaffected but sometimes cells probably of α_1 type might be encountered in which some of the numerous secretory granules exhibited a granule core with less electron density in the central part than in the peripheral rim (Fig 3) Diffusely scattered in the islets were cells of varying size showing cytoplasm of low electron density that often was devoid of secretory granules (agranular cells) The ductal ductular and acinar cells were unaffected There were no conspicuous signs of regeneration The nerve structures appeared to be normal

Relation Between the Duration of Diabetes and the Occurrence of Morphologic β Cell Changes

It was found that nuclear polymorphism and pyknosis as well as necrotic changes only occurred in some of the animals with diabetes of short duration (Nos 3 4 and 5) The other alterations showed no clear relation to the duration of the disease There were no morphological sex differences

DISCUSSION

A frequent light microscopic change in the diabetic Chinese hamsters was vacuolization of the pancreatic islet β cells This is a common diabetic lesion also in man and has often been given the name "hydro

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Fig 4

Pancreatic islet β cell (β) with dil t d end plasmic reticulum containing amorphous material (m) rather prom n nt (l i c mplex (C) and typical secretory granules (g) In another cell probably a / ll ac umulation of some fairly electron lucent material (F) is cer n h h a mitochon irion (M) can be recognized Animal No 7) × 1C 000

Fig ,

Islet β-cell in which gl en (Cl) seems to be deposited A few unaffe ted secretory granule (g) can a) be een in this cell (Animal No 16) × 18 000

6

7

TABLE 1
Survey of the Animal Material

Animal No	Sex	Age at the first appearance of diabetic symptoms (in months)	Age at sacrifice (in months)	Approximative duration of diabetic symptoms (in months)	Blood glucose level at the time of sacrifice (mg/100 ml)
1	♂	1	2	1	274
2	♂	1	2	1	437
3	♂	2	3	1	520
4	♂	1	3	2	317
5	♀	4	6	2	447
6	♂	1	4	3	310
7	♀	3	6	3	474
8	♀	3	7	4	338
9	♂	3	7	4	4 3
10	♀	9	14	5	342
11	♂	3	9	6	294
12	♀	3	10	7	215
13	♂	7	15	8	271
14	♀	2	14	12	234
15	♀	5	18	13	232
16	♂	6	21	15	290
17	♀	5	24	19	245

pic degeneration since the early description by *Weichselbaum & Stangl* (1901) It has been shown that the hydropic degeneration is due to cytoplasmic glycogen deposites (*Toreson* 1951) As glycogen was light microscopically demonstrated in the β cells in the present study it seems that glycogen infiltration may account at least for part of the vacuolization of these cells Another common diabetic alteration in man is decreased granulation of the β cells (*Bell* 1953) that has been found also in the Chinese hamster in previous (*Carpenter et al* 1967 *Malaisse et al 1967)* as well as in the present investigation Hyaline degeneration and fibrosis that often are found in the islets in states of established diabetes in human beings (*Ogilvie* 1964) were not found in the diabetic hamsters As hyaline change is rare in human juvenile onset diabetes and more frequent in the maturity onset type (*cf Warren & LeCompte* 1966) this denotes that the diabetes in the

Fig 6

Another β cell showing a cilium (Ci) with its basal body (b) close to the Golgi complex (G) The cilium protrudes into an intercellular space The relation between the ciliary and the cell membranes is clearly shown (Animal No 1) × 24 000

Fig 7

Islet tissue demonstrating typical β-cells (β) and cells (D) exhib[illegible] dense cytoplasm and a few secretory granules (g) seeming [illegible]
(Animal No 4) × 11 000

6

7

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7	♀	3	6	3	474
8	♀	3	7	4	338
9	♂	3	7	4	423
10	♀	9	14	5	342
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12	♀	3	10	7	215
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17	♀	5	24	19	245

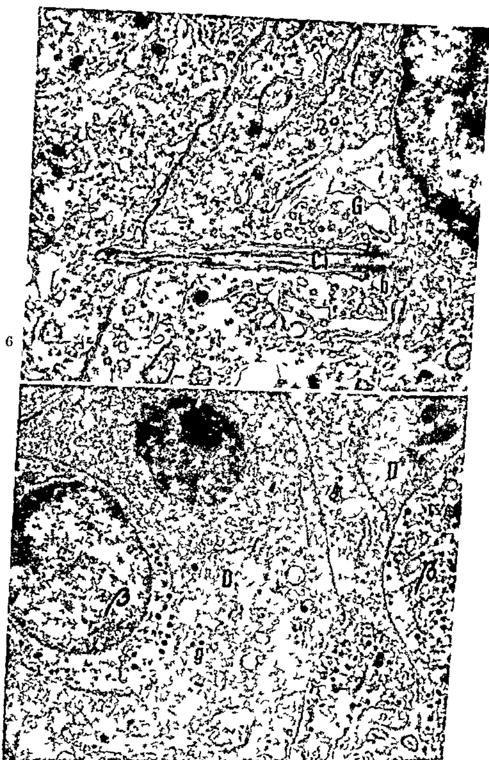
pic degeneration since the early description by *Weichselbaum & Stangl* (1901) It has been shown that the hydropic degeneration is due to cytoplasmic glycogen deposites (*Toreson* 1951) As glycogen was light microscopically demonstrated in the β cells in the present study it seems that glycogen infiltration may account at least for part of the vacuolization of these cells Another common diabetic alteration in man is decreased granulation of the β cells (*Bell* 1953) that has been found also in the Chinese hamster in previous (*Carpenter et al* 1967 *Malaisse et al* 1967) as well as in the present investigation Hyaline degeneration and fibrosis that often are found in the islets in states of established diabetes in human beings (*Ogilvie* 1964) were not found in the diabetic hamsters As hyaline change is rare in human juvenile onset diabetes and more frequent in the maturity onset type (cf *Warren & LeCompte* 1966) this denotes that the diabetes in the

Fig 6

Another β-cell showing a cilium (Ci) with its basal body (b) close to the Golgi complex (C) Th ill im p otru les into an intercellular space The rela ion between the cili ry and the cell membranes is clearly shown. (Animal No 10) X 24,000

Fig 7

I let ti su demon trating typical β-cells (β) and cells (D) exhibiting rather electron d n c toplasm and a few secretory granules (g) seemingly of β type (Animal No. 4) X 11 000


G
6
D
7

Chinese hamster in this respect rather is comparable to the human juvenile type. Though lymphocytes and plasma cells were scattered around the islets in one of the animals studied this alteration seemed to be too infrequent and inconspicuous to have a clear pathogenetic significance.

Also ultrastructurally there was decreased granulation of the β cells. Infiltrates of glycogen similar to those seen in other studies on spontaneous (*Williamson* 1960, *Like & Miki* 1967, *Luse et al.* 1967, *Patel et al.* 1967) and experimental (*Williamson & Lacy* 1961, *Volk et al.* 1965) diabetes in laboratory animals were found in the β cells of the diabetic hamsters. The significance of the accumulations of electron lucent material is not clear but they are probably of degenerative nature and possibly they represent fluid (*Williamson & Lacy* 1961). As these accumulations often were large, it seems that the cytoplasmic vacuoles seen in the light microscope can be due to deposits of both glycogen and electron lucent material. Dilated cisternae of endoplasmic reticulum containing amorphous material were observed also in tolbutamide treated rats where this material was thought to represent a precursor of a biologically inactive form of insulin (*Williamson et al.* 1961).

The β cells of the diabetic hamsters contained frequently various cytoplasmic bodies of uncertain significance. Some of these seemed to represent aggregates of secretory granules conforming to those seen in other endocrine cells (*cf. Smith & Farquhar* 1966). Others consisted of membrane enclosed particles that were reminiscent of secretory granules. The nature of these cytoplasmic bodies is unknown but they may possibly represent dilated cisternae of endoplasmic reticulum containing secretory material (*Schelin & Lundin* 1968). Such intracisternal secretory products may be regarded as signs of disturbed protein synthesis, stagnation of secretory products or enhanced secretion (*cf. Lundin & Schelin* 1964). The rather large cytoplasmic dense bodies have features in common with those of diabetic Chinese hamsters by *Luse et al.* (1967) stated to be lipofuscin. In the β cells of the present study were also myelin-like structures obviously denoting cellular degeneration (*Swift & Hruban* 1964).

F

l an at c i let β cell ng t ... glyc ( ) and dilated ... f the en l pla mi r t ... F B ... cell ar vari u st ... l l m many f ... Animal No 12 ... 000

f

A β cell ont in g ... gr ... pla mi l dies (a) sive of l ing aggregate of tw th ... Th e cytopla mi l d e wer ften ur nde l by membr n ... m) are also en to how m lin fig e an l th have m nil ... e lled aut ... g vacu les (l (Animal N 8 ... 000


ER
B
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Glycogen infiltration in diabetic states has been thought to be secondary to hypoinsulinaemia (*Duff & Toreson* 1951) or to hyperglycaemia (*Lazarus & Volk* 1958 *Williamson & Lacy* 1961 *Kern* 1968) *Meier & Yerganian* (1959) regarded it as a regressive response to excessive functional activity As this change has been found also in Chinese hamsters with hyperglycaemia induced by hydrocortisone but not in hydrocortisone treated animals without hyperglycaemia (*Boquist*, unpublished) it seems that this change in diabetes in this species rather is secondary to the hyperglycaemia Recently a close relationship has been shown to exist between the glycogen stores of the β cells and the glucose concentrations to which they are exposed (*Hellman & Idahl* 1968)

In the diabetic hamsters some cells probably of β type exhibited more electron dense cytoplasm than other β cells The significance of this cytoplasmic change is not known but it seems to be of interest that such cells have been found also in hamsters treated with growth hormone (*Boquist* unpublished)

Alloxan administration to laboratory animals can evoke pancreatic alterations conforming to those seen in spontaneous diabetes in laboratory animals and man (*cf Boquist* 1968 a) In the Chinese hamster β cell necrosis was occasionally found both after alloxan administration and in spontaneous hereditary diabetes The signs of regeneration seen in the alloxan treated animals were however not observed in the hamsters with spontaneous hereditary diabetes This may be a fact of decisive significance in the pathogenesis of diabetes mellitus in the Chinese hamster

From the data of previous studies on the Chinese hamster it has been concluded or suggested that the spontaneous diabetes occurring in this species is pancreatic in origin (*Yerganian* 1964 *Carpenter et al* 1967 *Gerritsen & Dulin* 1967 *Malaisse et al* 1967) In the present study the findings of β cell lesions give support to the concept that diabetes in the Chinese hamster is related to decreased β cell function and to deficient ability of the β cells to regenerate The possibility that extrapancreatic factors also may play a role can of course not be excluded by an investigation of the present kind

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[illegible] β [illegible] reticulum (ER) and [illegible] dense and rather homo[illegible] × 14 000

F [illegible] *11*

Anoth[illegible] mic accumulation of some [illegible] Some of the mitochondria [illegible] granules (arrows) [illegible] ance and electron density [illegible] ules are seen [illegible] 19 000

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Acta path microbiol scandinav 75 415–4?? 1969

The Department of Forensic Medicine University of Turku Turku 3 Finland
(Head Professor J rki Raekallio)

ALKALINE AND ACID PHOSPHATASE ACTIVITY IN THE INITIAL PHASE OF FRACTURE HEALING

By

JYRKI RAEKALLIO and PIRKKO LIISA MÄKINEN

Received 3 ix 68

The activity of several enzymes including phosphatases has been histochemically demonstrated to increase during the very first postoperative hours in healing skin wounds (9 10 11 12) With regard to fractures the view that an inert latent period occurs up to the second or third day after injury is still prevalent (2 3 6) This seems to be due to lack of investigations into the earliest phase of healing although numerous studies on alkaline phosphatase activity in later phases of fracture re pair have been performed (2 3 5 6 8 15) There have been but few reports on the distribution of acid phosphatase in bone regeneration and these studies have not involved the earliest phase (6 15) To elucidate the appearance of alkaline and acid phosphatase in the initial phase of fracture healing we made an experimental investigation on rats demonstrating the enzymes histochemically

MATERIAL AND METHODS

4 month old albino rats f b th sexes were used In ether anaesthesia the right tibia of each animal was fractur d in the mid diaphyseal region by digital pressure No attempt was made t immobilize the site of the injury or the animal Groups of three rats were kill d 1 4 8 10 1? and 16 hours and 1 2 and 3 days after fracturing

The fractured tibias were removed immediately after sacrificing and most of the s ft tissues were di se ted awa fr m the bones The unfixed tibias were deminera lized for 7? hour in 10 p r cent disod um ethylenediaminetetraacetate (EDTA) in 0 1 M pho phate b ff r t pH ? (1 14) The EDTA solution with the fractured b nes was kept at – 4 ( ) [illegible] circulated b a magnetic stirrer and changed every 24 h urs

The deminer liz [illegible] w fr zen on a block of dry ice attached to a chuck and cut at 14 µ [illegible] Th fr zen sections were placed on albuminized slides kept at room te [illegible] ient [illegible] dried in a cool air stream and fixed for 10 mi nutes in cold [illegible] 10 per cent formalin buffered with sodium acetate and adjusted t [illegible] e ti ns were washed, treated with 10 per cent EDTA for 20 minute [illegible] ratu e to ensure complete demineralization and re washed

Supported b [illegible] the *Sigri l Juselius Foundation* and from the *Finnish National Resea* [illegible] *r Medi al S iences*

The sections were further treated according to the following histochemical techniques alkaline phosphatase was visualized a) by the metal salt method of Gomori as described by *Burstone* (4) and in alternate sections b) by the azo dye method of Gregg and Pearse as presented by *Pearse* (7) Acid phosphatase activity was demonstrated c) by the metal salt method of Gomori as described by *Burstone* (4) and additionally d) by the azo dye method of Gregg and Pearse according to *Pearse* (7) The incubation times were as follows 90 minutes at + 37 C (method a) 45 minutes at room temperature (method b) 90 minutes at + 37 C (method c) and 45 minutes at room temperature (method d) Fast violet B and Fast garnet GBC were used as diazonium salts for the methods b and d respectively Control sections were incubated without the substrate. In addition alternate sections were stained by the van Gieson technique for histological study

RESULTS

The sites of alkaline phosphatase stained dark brown and those of acid phosphatase reddish brown by the azo dye methods Phosphatase activity appeared as a black stain in the Gomori type preparations In the uninjured bone (far from the fracture end) the cytoplasm of the osteoblasts and the vessel walls stained intensively and some osteocytes situated near the periosteum showed a weak to moderate reaction when the azo dye method for alkaline phosphatase was applied By the metal salt technique for the same enzyme also the nuclei of the osteoblasts and the collagen fibres took up the stain When the azo dye technique for acid phosphatase was applied osteoblasts stained intensively *and many cells in the bone marrow likewise took up the stain* By the metal salt technique for acid phosphatase diffusion artifacts disturbed the picture The enzyme pattern however resembled that demonstrable by the azo dye method

At one to eight hours after the injury the fracture defect was filled and surrounded by extravasated blood and inflammatory exudate Numerous polymorphonuclear leucocytes appeared in the exudate after four hours The cytoplasm of these cells was phosphatase positive by the azo-dye techniques In the Gomori type preparations also the nuclei took up the stain

At ten hours the first signs of increase in the phosphatase activity were noticed in the osteoblasts and undifferentiated osteogenic cells of the periosteum (Fig 1) Both acid and alkaline phosphatase activity of these cells (Figs 3 and 4) increased first in a peripheral zone This was situated at a distance of 200–500 μ and further away from the fracture line At 24 hours the osteoblasts and osteogenic cells also nearer the fracture line showed an intense acid and alkaline phosphatase activity (Fig 2) Between 10 and 12 hours the phosphatase activity of the

Figs 1–2

Fig 1 Acid phosphatase activity in a 10 hour fracture *The first signs of increase* in the enzyme activity are noted in the periosteal cell (Azo-dye method × 150)

Fig 2 Acid phosphatase activity in a 24 hour fracture The proliferating periosteal cells show an intense enzyme reaction (Azo dye method × 150) F = fracture line P = periosteum B = bone





Figs 3–4

Fig 3 Alkaline phosphatase activity in a 12 hour fracture Note the increased enzyme reaction of the periosteal cell in the peripheral zone situating at a distance of 200–500 μ and farther away from the fracture line (Metal salt method × 150)

Fig 4 The same fracture as in Fig 3 Alkaline phosphatase activity is demonstrated by using the azo dye method (× 180)



Fig 5
Alkaline phosphatase ... of p... te l cells in the peripheral zone of a 16-hour fracture. Initial prolif... [illegible] ... contributes to the intensification of enzyme activity (Azo ... e meth d × 360)

mentioned periosteal cells intensified but no cell proliferation could then be noticed At 16 hours (Fig 5) and subsequently an initial proliferative response was seen in the periosteum This occurred in the same peripheral zone which had showed the first increase in enzyme activity at ten hours The proliferation became more evident at 24 hours and during the second and third day after the trauma The proliferating periosteal cells showed an intense acid and alkaline phosphatase activity (Figs 2 and 3) Thus the general phosphatase activity of the periosteum in the peripheral zone increased from ten hours to about the second day and remained strong during the rest of the experimental period The first increase took place in the local periosteal cells After 16 hours the proliferation of these cells contributed to the intensification of enzyme activity Similar phenomena were noticed also in the endosteum

The intensification of enzyme activity could be demonstrated both by the azo dye and by the metal salt methods (Figs 3 and 4) The latter however were disadvantaged by diffusion artifacts

Three days after the injury the initial blood clot around the fracture ends was replaced by granulation tissue showing intense phosphatase activity The subperiosteal osteocytes which normally show a weak to

moderate alkaline phosphatase reaction had lost their enzyme activity at ten hours This decrease in alkaline phosphatase activity took place in a 200 to 500 μ deep central zone in the immediate vicinity of the fracture line The loss of enzyme activity became more evident later on and at 48 hours the karyolysis and karyorrhexis of the osteocytic nuclei could also be shown histologically After this time dead bone with empty lacunae characterized the central zone An irregular line of demarcation formed at a distance of 200 to 500 μ from the fracture line to separate the necrotic central zone from the living bone situated farther away from the fractured end However the periosteal and marrow tissues maintained their phosphatase activities almost undisturbed even nearer the fracture end and these tissues showed histological signs of necrobiosis in the immediate vicinity of the fracture line only

DISCUSSION

The decrease in alkaline phosphatase activity of the subperiosteal osteocytes in the central zone should be regarded as a sign of imminent necrosis demonstrable histochemically as early as ten hours after injury Necrobiosis and necrosis in this zone become visible histologically also two days after the trauma The diminishing vitality of the osteocytes in the immediate vicinity of the fracture line is considered to be principally due to the reduction of blood supply caused by local destruction and sealing off of the vessels It is interesting to note that the periosteal and marrow tissues having a better blood supply than the bone itself maintained their enzyme activity better even in the central zone No loss of acid phosphatase activity could be noticed in the osteocytes since these cells normally do not either show any demonstrable activity of this enzyme The loss of alkaline phosphatase activity in the osteocytes of the central zone resembles the loss of enzymes in the vicinity of the edge of healing skin wounds (9 10 11 12)

Far from being inert the early fracture healing is characterized by increasing acid and alkaline phosphatase activity located in the peripheral zone of periosteum and endosteum during the earliest days and even hours The initial increase in enzyme activity occurs in the osteoblasts and osteogenic cells at ten hours The proliferation of these cells after 16 hours further intensifies the phosphatase activity observed there By using tritiated thymidine the initial proliferative response to fracture has been seen at the same time (16)

The first reaction to fracture is essentially a sterile inflammation which is the consequence of trauma and haemorrhage (17) Also in the local periosteal cells there is a very early enzymatic response to injury demonstrable after 10 hours when the histochemical methods for acid and alkaline phosphatase are used Trauma affecting the multipotent periosteal cells in the peripheral zone seems to serve as a *stimulus* activating defense forces after a relatively short mobilization time

Thus the initial increase in enzyme activity probably represents an adaptive (inducible) defense mechanism by the local cells (9 10)

Besides functioning as a defense barrier the enzymes appearing in the peripheral zone may play a part in more specific regenerative processes The intervention of alkaline phosphatase in the formation of the protein matrix of bone appears to be accepted by most authors (13 18) It seems possible that acid phosphatase shares a role with alkaline phosphatase in the process of bone repair (15) Whatever the exact function of these enzymes *in vivo* it seems probable that the increase in enzyme activity represents one of the first steps in the repair process starting early in the so called latent phase of fracture healing

SUMMARY

The activity of acid and alkaline phosphatase was demonstrated histochemically in the healing experimental fractures of rats The animals were killed 1 2 4 8 10 12 and 16 hours and 1 2 and 3 days after fracturing the right tibia

The subperiosteal osteocytes had lost their alkaline phosphatase activity in ten days This decrease in enzyme activity took place in a 200 to 500 μ deep central zone in the immediate vicinity of the fracture line In the same area necrobiosis and necrosis could be histologically demonstrated in two days

In a peripheral zone farther away from fracture line the activity of acid and alkaline phosphatase began to increase ten hours after injury in the osteoblasts and osteogenic cells of the periosteum The proliferation of these cells after 16 hours further intensified the phosphatase activity there remaining strong during the rest of the experimental period Similar phenomena were observed in the endosteum

The decreasing enzyme activity in the central zone should be regarded as an early sign of imminent necrosis due to the reduction of blood supply The initial increase in enzyme activity in the peripheral zone probably represents an adaptive defense mechanism by the local periosteal and endosteal cells as an enzymatic response to injury Later on the enzymes may play a role in more specific regenerative processes

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The Enterovirus Department Statens Seruminstitut Copenhagen Denmark.

RUBELLA NEUTRALIZING ANTIBODY IN SERUM PRODUCTS

By

JØRGEN LEERHØY

Received 3 viii 68

Human serum products have been used for conferring passive immunity to women exposed to rubella during the first trimester of pregnancy and a high titre of rubella neutralizing (NT) antibody in the products intended for clinical use is obviously desirable The studies described in this report were undertaken to determine the potency of different serum products prepared from blood collected in Denmark with respect to the contents of rubella neutralizing antibody in order to establish a preliminary evaluation of their validity for rubella prophylaxis

MATERIALS AND METHODS

Neutralization (NT) tests were performed in rabbit cornea cells (SIRC) employing serial two fold dilutions of the sera starting at 1 4 In some of the experiments the NT titre was determined with the addition of 2 per cent normal guinea pig serum (GPS) to the medium used for dilution of sera and virus A detailed description of the NT test procedures has been published previously (3)

Haemagglutination inhibition (HI) tests were performed in the Takatsy micro titration system employing essentially the technique described by *Halonen et al* (2) All serum products examined were absorbed with kaolin to remove non specific inhibitors and 0 9 per cent saline made from demineralized water was used as diluent for serum and antigen The haemagglutinating rubella antigen was purchased from Orion OY Helsinki Finland Antibody titrations were performed employing serial two fold dilutions starting at 1 10

NT antibody titres have been calculated by the method of *Kärber* and expressed as the reciprocal of the initial serum dilution in the serum virus inoculum The HI antibody titres have been expressed as the reciprocal of the highest initial dilution of serum producing complete inhibition of haemagglutination Throughout this paper NT titres less than 4 and HI titres [illegible] than 10 have been recorded as 0. Unless otherwise stated all serum products [illegible] been examined unheated

Gamma globulin prepared from blood collected in Denmark was received from the Blood Fractionation Department of the Institute Three specimens of 10 per cent gamma globulin [illegible] examined [illegible] with the corresponding plasmas from which they were prepared [illegible] of 16 per cent gamma globulin were examined in NT tests with [illegible] the addition of 2 per cent GPS in accordance with the procedure [illegible] described (3) HI titres were also determined

Rubella [illegible] [illegible] preparations were obtained from the Serum Department of the Institute after approximately 6 months of storage at +4 C Th[illegible] which contained 0 01 per cent merthiolate were tested before and after dialysis

Human plasma Blood [illegible] [illegible] obtained from each of 4 blood donors [illegible] a past h[illegible] of rubella antibodies as well as heparin oxalate and citrate plasma

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TABLE 2

Rubella Neutralizing (NT) and Haemagglutination Inhibiting (HI) Antibody Titres in 16 per cent Gamma globulin Solutions

Material no	Antibody titres NT	NT (CPS)	HI
C161	64	512	640
C162	90	1024	640
C163	32	360	320

Neutralization test performed with 2 per cent guinea pig serum

Rubella Convalescent Serum

Two preparations of rubella convalescent serum were examined The preparations contained the preservative merthiolate which in preliminary experiments appeared to be toxic to the tissue cultures and part of the serum specimens were consequently dialysed before testing However the NT tests were difficult to read in dialysed as well as non dialysed sera Rubella NT antibody could not be ordinarily demonstrated in the two convalescent serum specimens tested and irregular results were obtained when NT tests were performed with the addition of 2 per cent GPS Thus in one of the specimens a titre of 6 was found in the dialysed serum and a titre of 0 in the non dialysed serum In contrast in the other convalescent serum antibody was not demonstrable in the dialysed serum but showed a titre of 16 in the non dialysed serum

Human Plasma

Human plasma prepared from blood treated with heparin oxalate or citrate was tested for rubella NT activity in order to investigate the possible influence of these commonly used methods for plasma preparation on the rubella NT antibody titre

The results of experiments performed on blood from 4 donors are shown in Table 3 It will be seen that rubella NT antibody titres of

TABLE 3

Rubella Neutralizing (NT) Antibody Titres in Serum and Differently Prepared Plasma

Material		NT				NT (CPS)			
Plasma	Heparin	16	4	2	64	23	45		[illegible]
	Oxalate	8	4	32	64	11	45	64	128
	Citrate	11	4	4	32	11	45	64	[illegible]
Serum		11	64	4	64	11	64	128	45
Donor no		1	2	3	4	1	2	3	4

Neutralization test performed with 2 per cent guinea pig serum

approximately the same level were found in the donor serum and in each of the different plasma preparations regardless of whether hepa rinized oxalate- or citrate blood was employed In some specimens a slight non significant enhancement of the titre values was seen when the NT tests were performed with the addition of 2 per cent normal GPS

Cohn Fractionated Material

Table 4 presents the results of NT and HI tests performed on three different lots of Cohn fractionated material The plasma NT titres of 16 to 45 are in agreement with titres commonly found in adult blood donors (5) In fraction I which mainly contains fibrinogen NT anti body could not be demonstrated in the only specimen available (lot C) In fraction II containing the gamma globulin rubella NT antibody was found in all three lots examined with titres ranging from 45 to 180 Fractions III and II + III were toxic to the cell cultures and the NT tests performed on these fractions could accordingly not be read Frac tion IV which mainly contains lipoprotein was available for 2 lots and showed a low NT antibody titre of 8 in lot A while no NT antibody could be demonstrated in lot C As regards lot B fraction IV was avail able as fraction IV-4 and IV green and NT antibody could be demon strated only in fraction IV green with a titre of 16 In the albumin containing fractions V and IV + V rubella NT antibody was not found

The results of the NT tests performed with the addition of 2 per cent normal GPS correspond with the results obtained in NT tests without GPS as far as demonstration of the presence or absence of antibody is

TABLE 4

Rubella Neutralizing (NT) and Haemagglutination Inhibiting (HI) Antibody Titres in 3 Lots of Cohn Fractionated Material

	Rubella antibody titres in Cohn fractionated material								
	lot A			lot B			lot C		
Material	NT	NT(GPS)	HI	NT	NT(GPS)	HI	NT	NT(GPS)	HI
Plasma	45	64	20	45	64	20	16	32	20
I	–	–	–	–	–	–	0	0	40
II	128	128	160	180	[illegible]60	320	4	90	320
III	ur	ur	80	ur	ur	160	ur	ur	80
II+III	ur	ur	160	ur	ur	320	–		
IV	8	32	40	–	–	–	0	0	20
IV-4	–	–	–	0	0	0	–		–
IV-green	–	–	–	16	128	80			
V	0	0	ur	0	0	ur	0	0	ur
IV+V	–	–	–	0	0	0	–	–	–

Neutralization test performed with 2 per cent guinea pig serum
ur = unreadable — = not done

concerned However the titre values are slightly higher in the plasma specimens and in fraction II of lots B and C In the two lots A and B where NT antibody is demonstrable in fractions IV and IV green a potentiating effect of GPS is seen with increases in the NT titres of 4 and 8 fold respectively

The results of the HI tests show that this type of antibody can be demonstrated in all Cohn fractions where NT antibody is present and further in the available fraction I and in fractions III and II + III In fraction IV of lot C HI antibody is demonstrable in contrast to the negative finding in the NT antibody test

DISCUSSION

Based on the assumption that human gamma globulin and rubella convalescent serum contain significant amounts of rubella neutralizing antibody administration of such preparations has for many years been generally recommended for treatment or prevention of rubella during pregnancy From a series of trials recently reviewed by *Green et al* (1) it appears however that the efficacy of such treatment by which to prevent rubella has been very variable Amongst several probable explanations of the variation in the protective value of gamma globulin *Schiff et al* (9) mention the difference in neutralizing antibody titre in several lots of gamma globulin assayed They suggest that gamma globulin intended for prophylactic use be selected on the basis of a high titre of rubella antibody This concept has been extended by our finding (4) that rubella patients develop maximal neutralizing antibody titres as late as 6 to 15 months after illness which suggests that blood intended for preparation of anti rubella serum products should be collected during this period

In the present study the rubella NT antibody titres of different serum and plasma products have been determined in order to establish a preliminary evaluation of their potency for rubella prophylaxis The plasma titres of 23-32 shown in Table 1 are in agreement with our previous finding of a mean rubella NT antibody titre of 16-23 in blood donors (5) The NT antibody titres of the corresponding 10 per cent gamma globulin solutions seem however to be slightly lower than could be expected from the concentration factor of 1 9 calculated on the basis of a 90 per cent purity of the gamma globulin and a content of 1 g gamma globulin per 100 ml of plasma In contrast to the expected increase in antibody titre of 9 fold the gamma globulins G1 and G3 only demonstrate a 4 fold increase from the titres found in the respective plasmas P1 and P3 while an 8 fold rise in titre is seen in the plasma and the corresponding gamma globulin set P2-G2 It is interesting to note that the HI titres for the same sets of preparations show a uniform increase of 8 fold closely corresponding to the calculated concentration factor

Acta path microbiol scandinav 75 430–434 1969

The Enterovirus Department Statens Seruminstitut Copenhagen Denmark

OBSERVATIONS OF THE STABILITY OF RUBELLA NEUTRALIZING ANTIBODY

By

JØRGEN LEERHØY

Received 15 viii 68

In a routine examination of sera stored at 4 C for approximately 2–3 weeks we found that rubella neutralizing antibody could not be demonstrated in a high proportion of the sera The sera were derived from pregnant women and according to a previous study of the frequency of rubella antibody in adults more than 90 per cent could be expected to be sero positive (6) The present study was therefore undertaken in order to investigate the possible influence of different storage and handling conditions on the stability of rubella neutralizing antibody

MATERIALS AND METHODS

Human sera In order to simulate the storage conditions of the sera employed in the above mentioned study two specimens of the same serum from each of 10 pregnant women were received from the Bloodgrouping Department of this institute The first specimen was stored at minus 20 C soon after the separation of the blood while *the second specimen was kept for 2 weeks at 4 C and subsequently stored at minus* 20 C until rubella antibody titres could be determined simultaneously

The influence of prolonged storage at room temperature and at 4 C respectively was studied in serum specimens from 2 adult blood donors with a previous history of rubella A number of test tubes each containing 0 7 ml of serum was stored at the respective temperatures and transferred to minus 20 C at the time intervals shown in Table 2 The specimens were kept in the dark during the experimental period in order to eliminate the possible influence of light The neutralization tests on these sera were performed with and without the addition of 2 per cent normal guinea pig serum to the medium used for diluting the virus and sera in accordance with procedures previously described (5)

The possible effect of repeated freezing and thawing was studied in sera from 3 adult blood donors Test tubes containing 0 6 ml of serum were frozen at approximately minus 50 C *in an alcohol dry ice bath and immediately thereafter thawed* in a water bath at approximately 14 C From each of the 3 blood donors serum specimens frozen and thawed 1 5 10 and 20 time were examined

The influence of lyophilization on rubella neutralizing antibody *titre was studied* in sera from 4 adult blood donors

Whole blood and serum from 3 donors were tested after shipment for 3 days by ordinary mail in order to compare the antibody titre in these samples *with specimens* of the same sera stored at minus 20 C

Neutralization tests were performed on rabbit cornea *cells SIRC) employing* serial two fold dilutions of the sera starting at 1 4 *A detailed description* of the test procedure has been published previously *(5) The sera examined* in the present study were not heat inactivated at 56 C The antibody titres have been calculated by the method of Karber *and expressed* as the reciprocal of the initial serum dilution in the serum virus inoculum Throughout this paper a titre less than 4 has been recorded as 0

RESULTS AND DISCUSSION

Immunoelectrophoresis of human sera have shown that certain fractions may be affected by storage (1 8 9) and as regards animal sera Kopp and Englert using paperelectrophoresis have reported that storage of pig serum at various temperatures between 6 C and 37 C gradually affected the percentual relation of the gamma globulin constituent (4) Observations of the instability of human gamma globulin preparations upon storage have also been reported by several investigators (2 3 10) and in a previous publication we reported the lack of demonstrable neutralizing antibody in 2 rubella convalescent sera stored at 4 C for 6 months (7)

TABLE 1
Rubella Neutralizing Antibody Titre in Sera Stored for 14 Days at 20 C and 4 C respectively

Patient no	Storage temperature	
	—20 C	4 C
1	11	8
2	32	23
3	16	23
4	11	0
5	23	90
6	0	0
7	64	23
8	32	0
9	16	0
10	16	0

The observations described in the present communication indicate that the rubella neutralizing antibody titre in human sera may be reduced by storage at 4 C in less than 14 days Table 1 shows the titres of 10 human sera stored for 2 weeks at minus 20 C and 4 C respectively Rubella antibody could be demonstrated in 9 of the sera stored at minus 20 C In 4 of the sera with titres ranging from 11 to 32 antibody could not be demonstrated after 2 weeks storage at 4 C while 5 sera titering from 11 to 64 did not show significant changes in titre In one serum rubella neutralizing antibody could not be demonstrated in neither one of the two serum specimens

The results of antibody titrations of 2 serum specimens after storage at room temperature or 4 C for various periods up to 56 days are recorded in Table 2 In serum from donor A antibody could not be demonstrated from day 14 in specimens stored at 4 C In the specimens stored at room temperature a variation in the antibody titre between 0 and 4 was seen from day 14 to day 28 whereafter antibody was no longer demonstrable

The antibody activity in serum samples from donor B did not show

sensitive staphylococci and strains of *Corynebacterium diptheriae Listeria monocytogenes and enterococci* that had been used in a previous investigation (*Wallerstrom* 1967)

Media For isolation and maintenance purpose the fungi were cultivated on Sabouraud agar (Oxoid)

For production of antibiotics a medium of the following composition (modif from *Palmstierna & Ripe* 1962) was used

casein hydrolysate (Difco)	1 *per cent*
glucose	1 per cent
NH_4NO_3	1 per cent
KH_2PO_4	0 5 per cent
$MgSO_4$ 7 H O	0 1 per cent

pH 5 One ml of a trace metal solution (see below) was added per 1 of medium This solution was prepared as follows CaCl 4 5 mMol $FeCl_3$ 61 f mMol $ZnSO_4$ 0 62 mMol $CuSO_4$ 0 62 mMol $MnSO_4$ 0 f6 mMol CoCl 0 77 mMol it was added to 60 mMoles of ethylene diamino tetra acetic acid suspended in 500 ml After dissolution by boiling 0 25 mMol of (NH) MoO was added and the solution was made up to 1000 ml Modifications of this standard medium were used in various experiments and are described in the text For some experiments use was made of a modified Czapek Dox substrate and malt peptone medium described previously (*Wallerstrom* 1967) and of beer wort prepared as described by *Hallmann* (1953) 0 1 per cent cycloheximide was added to all media used for production of antibiotics

In tests of the antibiotics activity of culture filtrates and EPF extracts Oxoid DST agar was used In some experiments the activity of EPF extracts was also tested on plates containing *Oxoid Blood Agar Base No 2 and 4 per cent horse blood* *Tube* dilution tests were carried out with bacteria growing in Oxoid s *Nutrient* Broth No 2

In order to eliminate penicillin which may be produced by strains of *Epidermophyton* (*Cole* 1966) Difco Bactopenase was added to the filtrates before extraction or to the Oxoid DST agar medium

METHODS

Cultivation for production of antibiotics The *Epidermophyton* strain were grown as described earlier (*Wallerstrom* 1967) in 500 ml flasks containing 250 ml culture medium at room temperature and 150 r p m on a rotary shaker

Determination of the weight of the mycelium (method modified from *Ziegler & Bohme* 1962) A number of 100 ml flasks containing 50 ml of medium were inoculated with washed mycelium from a culture on the same medium and cultivated during continous shaking At intervals of 24 hours one pair of flask after another was taken from the shaking machine and stored at —20 C At the end of the experiment all the cultures were thawed and filtered and the mycelium was dried for 24 hours at +60 C and weighed The mean of the growth of the two flasks harvested simultaneously was taken as the mycelial weight on that day

The pH of the culture media was measured with a Radiometer PHM with combi electrode

Addition of precursors to the cultures During the first *10* days of cultivation 0 5 ml of a 5 per cent solution in 99 8 per cent ethanol of each precursor was added daily to the fungal cultures Cultures to which had been added the corresponding amount of the solvent served as controls

Dialysis was carried out with 5 ml of EPF extract in dialysis tubing pore radius 24 Å (Union Carbide Co) against 50 ml of phosphate buffer pH 6 7 during stirring

Extraction Filtrates of culture medium were shaken with various organic solvents for 20-30 minutes at room temperature in a separation funnel with double the amount of the solvent The organic phase was separated from the water phase and evaporated at room temperature after which the residue was regenerated in distilled water to half the volume of the filtrate (in some instances one third) Unless otherwise stated the extraction was done at pH 7-8 If antibiotic activity was still demonstrable in the water phase after a single extraction the extraction was repeated twice

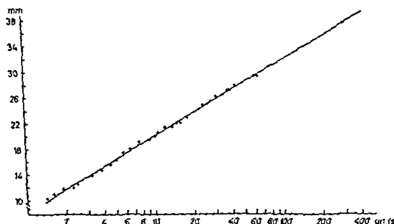


Fig 1
Zones in plate diffusion test and corresponding concentration of EPF in units per ml

The mycelia were dried for 24 hours at +60 C and crushed in a mortar 0 5 g of the powder was extracted by shaking with 50 ml of the solvent for 15 minutes The extraction was repeated twice evaporation was performed at room temperature and the residue was regenerated in 5 ml of distilled water

In the following the term extract is to be understood as diethyl ether extract of penicillinase treated filtered culture fluid

Testing the influence on EPF activity of heavy metal ions an cysteine 1 ml volumes of EPF extracts were incubated for 20 hours at +37 C in the presence of 1mg of the substance to be tested (*Burton & Abraham* 1951) It was determined that the same concentration of the substances had no effect on the test staphylococcus in agar plate diffusion test

Testing the influence of serum on EPF activity Plate diffusion tests were carried out with dilutions of an EPF extract on a series of agar plates to which had been added successively increasing amount of human serum (0 5–10 per cent) Each test was made four times Solutions of fucidin and kanamycin which are antibiotics with a strong and weak affinity to serum respectively (*Bulow Sorensen* 196 Gourevitch *et al* 1958) were used as controls Tube dilution tests were carried out with a set of tube containing 1 15 ml of nutrient broth and 0 15 ml of human serum 0 1 ml of an EPF extract was added to each tube and inoculation was performed with one loopful of an 20 hour broth culture of the test staphylococcu The tubes were incubated at +37 C for 20 hour Incubation for longer periods had no influence on the result

Definition of units of EPF Dilution f tw EPI extracts in narrow dilution intervals were tested four times with th t t tr in f *Staphyloc c us aureu* and the mean value f the inhibit r n) d f r each preparati n A dilu r producing an inhibition ne 0 mm n t t was rbitrarily state l to c t 10 units of EPI C n quentl f] bt ined when one extra t s diluted 1 39 and an th r 1) th t f EPI n the undiluted extra t ld be 390 and 90 unit p ml r | t)

S I 7

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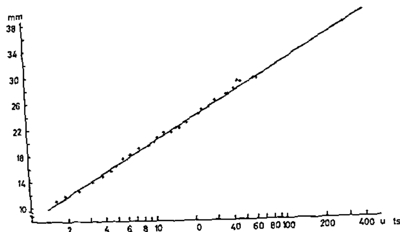


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Zones in plate diffusion test and corresponding concentration of FPF in units per ml

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Definition of units of EPF Dilutions of two EPF extracts in narrow dilution intervals were tested four times with the test strain of *Staphylococcus aureus* and the mean value of the inhibition zones was noted for each preparation A dilution producing an inhibition zone 20 mm in diameter was arbitrarily stated to contain 10 units of EPF Consequently if such a zone was obtained when one extract was diluted 1 38 and another 1 9 the content of EPF in the undiluted extract would be 380 and 90 units per ml respectively

RESULTS

Relationship between EPF Content and Inhibition Zones

By diluting two EPF extracts ol solutions with known amounts of EPF were prepared and these were tested in the plate diffusion test with the reference strain of *Staphylococcus aureus* The inhibition zone

produced by each solution was compared with its concentration of EPF The results are recorded graphically in Fig 1

For solutions containing less than 150 units of EPF the relationship between the logarithmus of the inhibition zones and the concentrations of EPF formed a straight line with an inclination angle of about 30 Solutions with higher concentrations of EPF gave comparatively smaller zones The standard deviation was $\sigma = \pm 0.58$ mm In another experiment each of a series of dilutions was divided into ten parts and stored at —20 C On consecutive days one part of each solution was thawed and tested with the agar diffusion test against the reference staphylococcus The inclination angel of the regression line was the same as in the above mentioned experiment the standard deviation was considerably higher $\sigma = \pm 1.81$ mm

Variation in EPF Producing Capacity between Recently Isolated Epidermophyton *Strains and Deterioration after Prolonged In Vitro Culture*

Epidermophyton strains vary in their capacity to produce antibiotics in a previous investigation 11 out of 50 recently isolated strains were classified as poor producers (*Wallerstrom* 1967) In cultures of a few recently isolated strains in this study the concentration of EPF never exceeded 10 units/ml The weight of the mycelium was determined in cultures of 4 low producing strains in two of them it was slightly above 60 mg/100 ml culture volume and in one it was 280 mg while one of the strains had an average value of 764 mg

Strains of *Epidermophyton* as well as other dermatophytes often cease to produce antibiotics when kept on Sabouraud agar The rate of deterioration of the EPF producing capacity was studied by repeated subculture of 9 strains of *Epidermophyton* Among 14 shake cultures performed after the fungi had been kept on Sabouraud agar for periods ranging between 2 and 6 months 8 contained less EPF than cultures grown within 1 month of isolation (*i e* produced a zone which was at least 5 4 mm smaller 5 4 = 3 σ) Four had the same and 2 had a larger EPF content When preserved in soil strains of *Epidermophyton* retained their ability to produce EPF better than when they were kept on Sabouraud medium 10 strains that had been stored in soil for from [illegible] to 15 month periods were recultivated on Sabouraud agar and 21 shake cultures were inoculated from these When compared to cultures of the same fungi grown within 1 month of isolation 6 had a smaller 9 had the same and 6 had a larger EPF content

Production of EPF in Relation to the Growth of the Fungi

30 cultures of 14 strains of *Epidermophyton* were followed for 3 weeks with daily determinations of the EPF concentration and hydrogen ion concentration of the culture fluid Detectable amounts of EPF

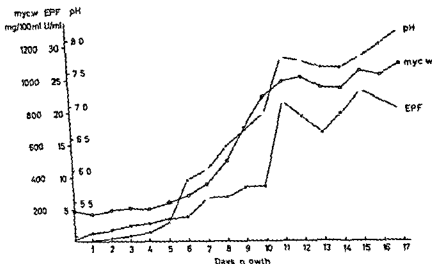


Fig 2

Concentration of EPF in the culture fluid in relation to the weight of the mycelium and pH

were found after culture for on the average 3 days (outer limits 1–9 days) There was no correlation between the duration of this lag phase and the final concentration of EPF but with freshly isolated strains EPF appeared earlier in the culture than with strains which were recultivated from stock cultures The maximum concentration of EPF was reached after 11 days (mean value) and only in a few cases was there any further increase later than 14 days after inoculation After having reached its maximum the EPF concentration usually remained stationary The maximum concentration of EPF in the medium in any member of this series was 120 units/ml

In experiments designed to determine the development of the mycelium the weight of the mycelium first increased rapidly and then leveled off The increase of EPF followed the increase of the mycelial weight with a lag of one or two days in most experiments When the weight of the mycelium had become stationary also the EPF content of the medium ceased to increase The mean maximum weight of the mycelium in these series was 1064 mg dry weight per 100 ml culture After growth for 5 11 days (mean 8 days) the weight did not increase further The pH of the medium ran fairly parallel to the curve for the weight of the mycelium rising from initially pH 4 7 to values between 7 5 and 8 (one typical experiment is depicted in Fig 2)

Variation of Cultures Technique

EPF is produced in small amounts presumably some tenths of mg per l culture (*Galenbeck & Wallerstrom* to be published) Two *Epider*

mophyton strains were therefore cultured in media other than the above described standard medium or on modifications of it or with slight modifications of the culture technique None of the variations resulted in an increase of the production of EPF

Inoculation 26 cultures were divided into three groups one group was inoculated with 0 1–0 2 cm of mycelial mat from growth of *Epidermophyton* on Sabouraud agar per 100 ml culture medium another group with 0 3–0 cm and a third group with 0 6–0 8 cm The mean EPF content of the culture fluid in the three groups after cultivation for 1 week was 28 7 43 0 and ?? units/ml respectively —Inoculation with material from cultures grown on the standard medium did not give better results than when material from growth on Sabouraud agar was used

Carbohydrate variations Increase of the glucose concentration in the medium to 8 per cent resulted in an earlier onset of the production of EPF but after 6 days cultivation the amount of EPF equalled that in the control Nor were better results obtained when lactose maltose or mannitol were used in place of glucose or when 1 3 per cent corn steep liquor or molasses was added to the medium

Nine cultures were grown on a medium consisting of a salt solution (the mixture used in the standard medium) and 3 or 8 per cent glucose The fungi developed slower in these than in control cultures and after growth for 3 weeks the EPF content of the culture medium was 5–25 units/ml There was a slight fall of the pH in the medium which was most marked in cultures with 8 per cent glucose

Other media Cultures on Sabouraud broth Czapek Dox broth malt peptone broth and beer wort did not yield more EPF than did cultures on the standard medium

Addition of precursors In view of an assumed relation between EPF and antibiotics with a steroid skeleton (Calenbeck & Wallerström to be published) substances involved in steroid synthesis were added During the first 10 days of growth small amounts of mevalonic acid lactone squalene lanosterol and cholesterol were added daily to a final concentration of 1 mg per ml of medium The cultures were grown for 3 weeks but the production of EPF in these cultures and the controls was not found to differ

Cultivation without agitation For comparison a strain of *Epidermophyton* was grown in stationary culture in five 500 ml flasks containing 250 ml of the standard medium After 9 days the EPF concentration was 10 units/ml in one of these in the others the yield was smaller or no antibiotic activity could be demonstrated The fungus grew in floccules on the bottom of the flasks with little tendency to surface growth

Extraction and some Chemical and Physical Properties of EPF

Extraction of EPF from the culture media proved possible with diethyl ether isopropyl ether chloroform butyl and ethyl acetate and benzene Extraction was not successful with hexane or petroleum ether From dried mycelium EPF was extracted also with ethanol acetone and dioxane the substance is soluble also in dimethyl sulphoxide (DMSO) From the amount of EPF in the culture fluid 25 40 per cent was transferred to the organic phase by shaking with chloroform or diethyl ether for 30 minutes at pH 7–8 but with ethyl acetate apparently all of the antibiotic was taken up by the solvent in the same time

At pH levels between 5 and 8 the transfer of EPF to the organic phase was largely the same but after further alkalinization some activity remained in the water phase (Table 1) After acidification the amount of EPF in the organic phase was smaller although no activity was found in the water phase This is explained by the fact that EPF is

TABLE 1

Ether Fxtraction of EPF from Culture Fluid at different pH Ievels

pH of fluid	zone diameter (in mm) organic phase	water phase	approximate amount of FPF taken up by solvent (per cent)
?	20	no z)ne	6
4	24	no zone	15
5	2f 5	n(zone	??
6	?7	no zone	?5
7	27	n) zone	25
8	?7	(+)	25
9	2?	?8	10
10	??	27	10

Zone before extra tion 28 5 mm (agar plate diffusion test)
10 ml of culture flui l was shaken v ith 20 ml of ether After evaporation of the s lvent the re idue was regenerated in 3 ml of water

only slightly soluble in water and presumably still less so at acid pH (*Gatenbeck & Wallerstrom* to be published)

The results obtained by *extraction of EPF from mycelium* suggested the existence of only a small amount of the antibiotic in this part of the culture Fxtracts of 500 mg of dried mycelium from each of 14 cul tures whose culture fluids contained 21–38 units of LPF per ml was carried out by repeated shaking with acetone After drying the sub stance was regenerated in ɔ ml of water The zones in the subsequent plate diffusion test indicated the presence of 30–180 units of I PF per g dry mycelium As the content of mycelium in a culture is about 1000 mg per 100 ml the amount of EPF in the mycelium in these cultures was no more than a few per cent of the total amount of FPF in the culture I PF w is found to be at least as soluble in acetone as in ether as judged from experiments where the residue of an ev apor ited filtrate was extracted with each of these solvents

Dialysis of I PF extracts against phosphate buffer pH 6 7 showed the subst ance to be dialyzable the concentr ation of I PF being equi librated within 24 hours

TABLE

Effect of Heating EPF Extract ... 100 ... at ... pH Levels Zone Diameter in the Plat Diffu ... Test ... after Treatment

pH	time (min)	[illegible] (mm)	[illegible] (mm)	activity diminished by (per cent)
	(	0 5	0 5	
5	0	20 2	19 7	0
9	(	21 5	1 7	0
1		13	19	0
12	1(	9	19	70
1		(+)	19	85
				ad 100

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Inoculation. 26 cultures were divided into three groups: one group was inoculated with 0.1–0.2 cm² of mycelial mat from growth of *Epidermophyton* on Sabouraud agar per 100 ml culture medium, another group with 0.3–0.5 cm², and a third group with 0.6–0.8 cm². The mean EPF content of the culture fluid in the three groups after cultivation for 1 week was 23.7, 13.0 and 20.5 units/ml respectively.—Inoculation with material from cultures grown on the standard medium did not give better results than when material from growth on Sabouraud agar was used.

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9	22	28	10
10	[illegible]	27	10

Zone before extraction 28 5 mm (agar plate diffusion test)
10 ml of culture fluid was shaken with 20 ml of ether After evaporation of the solvent the residue was regenerated in ml of water

only slightly soluble in water and presumably still less so at acid pH (*Gatenbeck & Wallerstrom to be published*)

The results obtained by extraction of EPF from mycelium suggested the existence of only a small amount of the antibiotic in this part of the culture Extracts of 500 mg of dried mycelium from each of 14 cultures whose culture fluids contained 21–38 units of EPF per ml was carried out by repeated shaking with acetone After drying the substance was regenerated in 5 ml of water The zones in the subsequent plate diffusion test indicated the presence of 30–180 units of EPF per g dry mycelium As the content of mycelium in a culture is about 1000 mg per 100 ml the amount of EPF in the mycelium in these cultures was no more than a few per cent of the total amount of EPF in the culture EPF was found to be at least as soluble in acetone as in ether as judged from experiments where the residue of an evaporated filtrate was extracted with each of these solvents

Dialysis of EPF extracts against phosphate buffer pH 6 7 showed the substance to be dialyzable the concentration of EPF being equilibrated within 24 hours

TABLE 2

[illegible] Ext[illegible] 100 [illegible] at Various pH Levels Zone Diameter in the [illegible] Diffusion Test before and after Treatment

pH	time min	test tube (mm)	control tube (mm)	activity diminished by (per cent)
2	[illegible]	20 5	20 5	0
5	[illegible]	20 2	19 7	0
9	[illegible]	21 5	21 7	0
[illegible]	5	13	19	70
[illegible]	10	9	19	85
12	[illegible]	(+)	19	ad 100

The termoresistance of the substance was studied at different hydrogen ion concentrations (Table 2). At pH below 9 no deterioration of the FPF was observed after boiling for 20 minutes; at pH 12 boiling for only 5 minutes reduced the activity by about 70 per cent. In another experiment it was found that at neutral reaction boiling for 30 minutes reduced the activity by 70–90 per cent and after boiling for 60 minutes no antibiotic activity remained in the solutions. Incubation overnight at 37 °C at pH levels varying between 1 and 12 did not reduce the activity of a series of FPF extracts. Addition of cysteine or heavy metal salts ($CuSO_4 \cdot 5\,H_2O$, $Pb(NO_3)_2$, $HgCl_2$, $AgNO_3$, $ZnSO_4$) had no effect on the antibiotic activity.

TABLE 3

Effect on Activity of EPF of Addition of Serum to the Test Medium in the Tube Dilution Test

Conc of EPF in medium	Growth in nutrient broth	Growth in nutrient broth + 10 per cent serum
8 units/ml	0	0
5.3 units/ml	0	0
4 units/ml	0	0
3.2 units/ml	0	+
2 units/ml	0	+
1 unit/ml	0	+
0.5 units/ml	+	+
0.25 units/ml	+	+
0.125 units/ml	+	+
Control	+	+

0 = no visible growth   + = growth within 20 h/37 °C

TABLE 4

Effect on Activity of FPF of Addition of Serum to the Test medium Zones in the Plate Diffusion Test

Antibiotic	Zone (mm) with serum concentration 0%	0.5%	1%	5%	10%	Zone on blood agar
FPF 56 U/ml	29.0	27.5	25.5	19.5	17.0	23.4
FPF 30 U/ml	26.0	24.8	23.4	18.1	16.0	20.8
FPF 20 U/ml	24.0	23.8	22.8	16.8	14.0	18.8
Fucidin 50 mcg disc (control)	34.0	33.8	30.8	23.8	19.8	
Kanamycin 5 mcg disc (control)	17.0	16.8	16.8	17.0	17.0	

Studies on the activity of FPF were performed after addition of serum to the test medium. In tube dilution tests with 10 per cent human serum present the minimal inhibitory concentration of FPF was at least 4 times that of the control. The results of one typical experiment are given in Table 3.

Similar results were obtained in plate diffusion tests where it was found that the diameters of the inhibition zones varied inversely with the concentration of serum in the medium (Table 4) The zones produced by fucidin but not those produced by kanamycin were also smaller as expected by the affinity of these antibiotics for serum When the plate diffusion tests with LPF were performed on blood agar the zones were smaller than when the test was carried out on DST agar The difference corresponded roughly to the difference in their content of serum (Table 4)

In another experiment some LPF extracts were mixed with serum and tested in plate diffusion tests The diameters of the inhibitory zones were much smaller than diameters observed when the same extracts were mixed with the same amount of physiological saline (Table 5)

TABLE 5

Effect on Activity of LPF of Mixing Extracts with Serum before Plate Diffusion Test

Preparation labelled	Zone diameter (in mm) of extract untreated	extract mixed 1 1 with phys saline	extract mixed 1 1 with serum
A	24	19	15
B	30	[illegible]	17
C	32	9	

The antibiotic activity of LPF increased with the hydrogen ion concentration of the medium In plate diffusion tests at pH 7 the zones were smaller than at pH 5 and on alkaline medium the zones were still smaller (Table 6) In tube dilution tests at pH 7 the m i c was at least 4 times as high as at pH 5 (Table 7)

TABLE 6

Effect on Activity of LPF of Variation in pH of the Test Medium
Tube Dilution Test

Conc of LPF in test medium (units/ml)	pH of the test medium			
		7	8	9
4	0	0	0	0
2	0	0	0	0
1	0	0	0	0
0 5	0	+	+	+
0 25	0	±	+	±
0 125	−	+	+	+
Control		+	−	+

0 = no visible growth   + = growth after 20 h/37 C.

TABLE 7

Effect on Activity of EPF of Variations in pH of the Test Medium Zones in the Plate Diffusion Test

Preparation No	Concentration of EPF (units/ml)	Inhibition zone (in mm) at pH 5	7	9
1	3	18.5	14	9
2	12	24	21	13.5
3	12	23	21	11
4	21	26	24	14
5	31	27	26	13.5

DISCUSSION

With the present method of culture the Epidermophyton factor (EPF) was obtained only in minute amounts. Its appearance in the culture medium was closely connected with the growth of the mycelia particularly with the period of most rapid development the trophophase (*Bu'Lock et al* 1965). In this respect it differs from most other antibiotics which are produced in the idiophase as secondary or shunt metabolites derived from abnormal concentrations of cellular constituents in growth limited cultures (*Woodruff* 1966).

Poor production of EPF in cultures of recently isolated strains was most often associated with poor growth; these strains thus seemed to be unfit for the culture medium. On the other hand, strains that grew well and originally were good antibiotic producers on repeated subcultivation gradually produced less EPF though the weights of the mycelia remained normal. The tendency of *Epidermophyton* strains to lose their antibiotic production was reported by *Ura et al* (1952), Nishio (1958) and *Katagiri* (1967). *Urabe* (1951) found that the loss was associated with the phenomenon of pleomorphic degeneration. In the present study early pleomorphic degeneration was not accompanied by cessation of EPF production but old pleomorphic strains of *Epidermophyton* produced hardly any antibiotic.

EPF was produced by *Epidermophyton* on a variety of media independently of carbohydrates. Most of the antibiotic was released from the mycelium and was found in the culture fluid. Agitated cultures were better than non agitated cultures for the production. EPF also was formed in cultures on media consisting of glucose and minerals. In these cultures there was no alkalinization of the medium, a phenomenon accompanying growth of dermatophytes on tissue extract media and attributed to deamination of amino acids by the fungi (*Burrack & Knight* 1958).

EPF was extracted from medium and mycelium by means of several organic solvents and best at pH 7–8. The antibiotic activity was rather thermostable and not affected by variation of the pH between 2 and 12. However, it was destroyed by boiling at strong alkaline pH. EPF was

most active in an acid milieu as shown by lower m i c in the tube dilution tests and the production of larger inhibition zones in the plate diffusion tests

EPF was markedly less active in the presence of serum In this respect it is like fucidin (*Bulow Sorensen* 1962) EPF and fucidin have several other properties in common both are more active in acid media and both have a similar narrow antibacterial spectrum with a characteristic difference in activity between near related bacteria such as staphylococci and streptococci or corynebacteria and *Listeria* (*Bulow Sorensen* 1962 *Wallerstrom* 1967) Some staphylococcal strains resistant to EPF were found to be resistant also to fucidin (*Wallerstrom* 1967) Cross resistance among bacteria has been demonstrated for fucidin and cephalosporin P_1 (*Barber & Waterworth* 1962) and for the latter antibiotic and helvolic acid (*Burton & Abraham* 1951) The relation of EPF to these substances will be the subject of further study

SUMMARY

30 strains of *Epidermophyton floccosum* were cultivated submerged in agitated cultures and investigated for their production of an antibiotic the Epidermophyton factor (EPF) The antibiotic accumulated most rapidly in the culture fluid during the period of most active growth of the mycelium The amount of EPF in the mycelium was much smaller than in the culture filtrate EPF was produced on a variety of media even on a mixture of glucose and salts It was extracted from the mycelium and from the culture filtrate by means of several organic solvents It was dialysable rather thermostable and stable within wide ranges of pH Its antibacterial activity was reduced by the presence of serum and increased by acidifying the test media The antibacterial spectrum and other properties of EPF are similar to those of fucidin

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TABLE 7

Effect on Activity of EPF of Variations in pH of the Test Medium Zones in the Plate Diffusion Test

Preparation No	Concentration of EPF (units/ml)	Inhibition zone (in mm) at pH 5	7	9
1	3	18.5	14	9
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1	3	18 5	14	9
2	12	24	21	13 5
3	12	23	21	11
4	21	26	24	14
5	31	27	26	13 5

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TABLE 2
Serological Surveys of Cats

Author	Country	No of cats	Dye test pos	C F test pos	Skin test pos	Titre ≧
Miller & Feldman 1953	U S A	44	21(47 7)	–	–	1 4
Morris et al 1956	U S A	8	–	0	–	1 8
Feldman & Miller 1956	U S A	35	11(31)	–	–	1 16
Jones et al 1957	U S A	175	100(57 2)	–	–	1 16
Gibson & Eyles 1957	U S A	35	29(83)	–	–	1 16
Roever Bonnet 1958	Holland	13	5(38 5)	–	–	1 16
Eyles et al 1959	U S A	282	138(48 9)	–	–	1 16
Cook & Pope 1959	Australia	29	–	0	–	1 8
McCulloch et al 1964	U S A	21	1(4 8)	–	–	1 16

Figures in brackets percentage

MATERIAL AND METHODS

During the period July 1st 1959 to July 26th 1960 blood samples were received from 1000 dogs and 192 cats admitted to the Small Animal Clinic at the Royal Veterinary and Agricultural College in Copenhagen The sera were originally collected for a study of the incidence of leptospirosis in Denmark (*Borg Petersen & Fennestad* 1962 1967) and were kindly submitted for the present study

At the time of admission information was recorded about age and sex of the animals together with the address of the owners 46 sera from dogs and 8 sera from cats were excluded from the material due to the incompleteness of that information

Of the remaining 954 dogs 481 were males and 473 were females 734 came from the Copenhagen area while 220 were from outside that area (mainly Zealand)

Of 184 cats 64 were males and 120 females Grouping according to geographical origin was not possible since only a small number of the animals came from areas outside Copenhagen

The sera were stored in a freezer at —20 C from the time of collection until the end of 1966 when they were examined by the Sabin Feldman dye test The test was performed as described by *Sabin & Feldman* and as standardized for routine use in this laboratory by *Aagaard* All sera were inactivated for 30 minutes at 56 C, and all titres reported are final dilutions titres In this laboratory a titre of ≧ 1 10 in human sera is considered to be positive

Post mortem examination of dogs that died during the admission was performed by Tage Møller D V M who will present a detailed description in a separate paper

RESULTS

Dogs

A *total* of 954 dogs were examined serologically (Table 3) 548 sera (57 5 per cent) were negative with a dye test titre <1 10 406 sera (42 5 per cent) were positive with a titre of 1 10 or higher 207 (21 7 per cent) had titres of 1 10 166 (17 4 per cent) 1 50 and 28 (2 9 per cent) 1 250) Five sera (0 5 per cent) had a titre of 1 1250

A distribution according to sex is also presented in Table 3 Of 481 *males* 275 (57 2 per cent) were negative and 206 (42 8 per cent) positive 106 (22 0 per cent) had titres of 1 10 76 (15 8 per cent) 1 50 and 20 (4 2 per cent) 1 250 Four sera in this group were found to have a titre of 1 1250

TABLE 1
Serological Surveys of Dogs

Author	Country	No of dogs	Dye test pos	C F test pos	Skin test	Titre ≧
Siim 1950	Denmark	54	10(18)	–	–	1:250
Olten et al 1950	Germany	84	30(36)	–	–	1:25
Miller et Feldman 1953	U S A	51	34(66.6)	–	–	1:1
Cole et al 1953	U S A	1944	–	–	76(3.9)	–
Cole et al 1953	U S A	51?	0	–	–	
Feldman et Miller 1956	U S A	125	51(40.8)	–		1:16
Fainson 1956	England	113	–	48(42.5)		1:8
Morris et al 1956	U S A	180	–	45(25)	–	1:8
Borgen et Berg 1957	Norway	200	89(44.5)	–	–	1:4
Angeloff et al 1957	Bulgaria	45	–	–	6(13.3)	–
Gibson et Eyles 1957	U S A	9	3(33.3)	–	–	–
Roever-Bonnet 1958	Holland	75	6(8)	–	–	1:16
Cook et Pope 1959	Australia	30	–	2(7)	–	1:8
Seeman 1959	Czechoslovakia	34	–	6(18.0)	–	1:16
Rohde 1959	Germany	203	38(18.7)	–	38(19.7)	1:4
Eyles et al 1959	U S A	800	127(16)		–	1:16
Gibson et Jumper 1960	U S A	137	62(45.3)	–	–	1:4
Gibson et Jumper 1960	U S A	663	106(16.0)	–	–	1:16
Sato 1960	Japan	69	19(27.5)	–	–	1:16
Bosch et Rommel 1963	Germany	929	809(87.1)		–	1:4
Bosch et Rommel 1963	Germany	729		159(21.8)	–	1:5
McCulloch et al 1964	U S A	11	3 (33.0)		–	1:4
Kuil 1966	Dutch Guiana	137	51 (33)	–	–	1:16
Roever-Bonnet 1967	Dutch Guiana	70	14(20)	–	–	1:16

Figures in brackets percentage

to 47 2 per cent Similar results were obtained by *Lainson* (1956) who found 33 3 per cent of 24 London dogs under one year of age with complementfixing antibodies as against 45 per cent of 89 dogs over one year of age The sera were tested in dilution 1 8 only *Gibson & Jumper* (1960) found that 8 1 per cent of 272 juvenile dogs had antibodies with dye test titres of 1 16 or higher as against 19 9 per cent of 528 adult dogs However they had no information about the age of the dogs and the distinction between adult and juvenile dogs was based on size in relation to breed and on development of the genitalia Also *Boch & Rommel* (1963) found the rate of infection to be lower among younger than among older dogs Of 41 dogs below 6 months of age 63 4 per cent were positive whereas 87 3 per cent of 71 dogs from 6 months to one year old were positive with titres in the dye test of 1 4 or higher

This difference in infection rate between young and old dogs might be due to the fact that older dogs have had more time to acquire the infection and produce antibodies A different explanation is offered by *Lainson* (1956) who emphasizes that young dogs are more susceptible to toxoplasma infection than older dogs He suggests that because of this the likelihood of detecting toxoplasma antibodies among the lower age group is considerably diminished since young infected animals usually die and are consequently not available for serological study Whether this is correct must depend to a large extent also on the strain of toxoplasma involved

In Table 5 the dogs are divided into two groups according to their geographical origin 220 dogs came from outside the greater Copenhagen area and 734 dogs from within that area It should be pointed out that this is not a distinction between urban and rural dogs The difference between these two groups is merely geographical and the conditions under which the dogs are kept are probably fairly uniform These facts are reflected in the figures which reveal no difference in the percentage of serologically positive individuals in the two groups

Cats

It might have been expected that cats would be infected more frequently than dogs in the same area The cat is more of a hunter than the dog and its diet is less well controlled They may kill and eat a wide variety of birds rats and mice This is true not only of stray cats but also of family pets of which the present material is composed

Of 184 cats 62 5 per cent were found to possess toxoplasma antibodies Whether that percentage is representative for this area might be questioned Firstly the number of cats examined is much smaller than the number of dogs and [illegible] all the cats included in this material were brought to the Small Animal Clinic for veterinary treatment which means that they are all family pets No stray animals have been examined and as already pointed out one might expect an even higher percentage of serologically positive individuals in that group

TABLE 7

Serological Examination of 184 Cats Grouped According to Age

Age (years)	No	Negative <1 10	Positive ≧1 10	Dye test titres			
				1 10	1 50	1 250	1 1250
≦1	79	33(41 8)	46(58 2)	8(10 1)	29(36 7)	8(10 1)	1(1 3)
>1	96	29(30 2)	67(69 8)	26(27 1)	34(35 4)	7(7 3)	-

Figures in brackets percentage

(30 2 per cent) were negative and 67 (69 8 per cent) positive 26 of these (27 1 per cent) had a titre of 1 10 34 (35 4 per cent) had 1 50 and 7 (7 3 per cent) had 1 250

DISCUSSION

Dogs

The results of this study show that a considerable number of dogs in the Copenhagen area are serologically positive to toxoplasmosis 42 5 per cent being found to have antibodies It is believed that the figures are representative for this area (a) because they are based on a relatively large number of dogs and (b) because dogs in this area are kept under fairly uniform conditions Almost all are family pets stray dogs being rare Their access to wild life must be considered to be rather limited but an important source of infection might be raw meat Pork and mutton have been demonstrated frequently to contain toxoplasma (*Work* 1967 1968) and many dogs are regularly given raw meat Another source of infection might be toxoplasma infected nematode eggs However as already pointed out the frequency of this mode of transmission in nature is unknown and further studies are required to estimate its importance

No difference could be demonstrated in the infection rates of males and females Antibodies were found in 42 8 per cent of the males in 42 3 per cent of the females Nor was any difference observed between the sexes with regard to distribution of the titres This confirms similar observations of *Lainson* (1956) and *Gibson & Jumper* (1966)

Since the information about the age of the dogs in this study was fairly accurate different age groups were compared with regard to infection rate In former studies on pigs (*Work* 1967) the number of serologically positive individuals was higher among older than among younger animals In the present material 31 2 per cent of dogs up to 6 months of age were positive whereas in the age group from 6 months to 2 years 43 1 per cent had antibodies Statistical evaluation[1] showed this difference to be only weakly significant The rate of infection was fairly constant among dogs more than 6 months old varying from 40 ,

[1] The author is indebted to S Olesen Larsen M A Biostatistical Department for statistical evaluation of the results

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In Table 5 the dogs are divided into two groups according to their geographical origin 220 dogs came from outside the greater Copenhagen area and 734 dogs from within that area It should be pointed out that this is not a distinction between urban and rural dogs The difference between these two groups is merely geographical and the conditions under which the dogs are kept are probably fairly uniform These facts are reflected in the figures which reveal no difference in the percentage of serologically positive individuals in the two groups

Cats

It might have been expected that cats would be infected more frequently than dogs in the same area The cat is more of a hunter than the dog and its diet is less well controlled They may kill and eat a wide variety of birds rats and mice This is true not only of stray cats but also of family pets of which the present material is composed

Of 184 cats 62 5 per cent were [illegible] to possess toxoplasma antibodies Whether that perc[illegible]sentative for this area might be questioned Firstly the [illegible] examined is much smaller than the number of dogs an[illegible] all the cats included in this material were brought to the S[illegible]mal Clinic for veterinary treatment which means that they [illegible]mily pets No stray animals have been examined and [illegible] already [illegible] out one might expect an even higher percentage of serologic[illegible]sitive individuals in that grou

TABLE 7

Serological Examination of 184 Cats Grouped According to Age

Age (years)	No	Negative <1:10	Positive ≧1:10	Dye test titres 1:10	1:50	1:250	1:1250
≦1	79	33(41.8)	46(58.2)	8(10.1)	29(36.7)	8(10.1)	1(1.3)
>1	96	29(30.2)	67(69.8)	26(27.1)	34(35.4)	7(7.3)	-

Figures in brackets: percentage

(30.2 per cent) were negative and 67 (69.8 per cent) positive; 26 of these (27.1 per cent) had a titre of 1:10, 34 (35.4 per cent) had 1:50 and 7 (7.3 per cent) had 1:250.

DISCUSSION

Dogs

The results of this study show that a considerable number of dogs in the Copenhagen area are serologically positive to toxoplasmosis, 42.5 per cent being found to have antibodies. It is believed that the figures are representative for this area (a) because they are based on a relatively large number of dogs and (b) because dogs in this area are kept under fairly uniform conditions. Almost all are family pets; stray dogs being rare. Their access to wild life must be considered to be rather limited, but an important source of infection might be raw meat. Pork and mutton have been demonstrated frequently to contain toxoplasma (*Work* 1967, 1968) and many dogs are regularly given raw meat. Another source of infection might be toxoplasma-infected nematode eggs. However, as already pointed out, the frequency of this mode of transmission in nature is unknown and further studies are required to estimate its importance.

No difference could be demonstrated in the infection rates of males and females. Antibodies were found in 42.8 per cent of the males, in 42.3 per cent of the females. Nor was any difference observed between the sexes with regard to distribution of the titres. This confirms similar observations of *Lainson* (1956) and *Gibson & Jumper* (1960).

Since the information about the age of the dogs in this study was fairly accurate, different age groups were compared with regard to infection rate. In former studies on pigs (*Work* 1967) the number of serologically positive individuals was higher among older than among younger animals. In the present material 31.2 per cent of dogs up to 6 months of age were positive, whereas in the age group from 6 months to 2 years 43.1 per cent had antibodies. Statistical evaluation[1] showed this difference to be only weakly significant. The rate of infection was fairly constant among dogs more than 6 months old, varying from 40.5

[1] The author is indebted to S. Olesen Larsen, M.A., Biostatistical Department, for statistical evaluation of the results.

to 47.2 per cent. Similar results were obtained by *Lainson* (1956) who found 33.3 per cent of 24 London dogs under one year of age with complementfixing antibodies as against 45 per cent of 89 dogs over one year of age. The sera were tested in dilution 1:8 only. *Gibson & Jumper* (1960) found that 8.1 per cent of 272 juvenile dogs had antibodies with dye test titres of 1:16 or higher as against 19.9 per cent of 528 adult dogs. However they had no information about the age of the dogs and the distinction between adult and juvenile dogs was based on size in relation to breed and on development of the genitalia. Also *Boch & Rommel* (1963) found the rate of infection to be lower among younger than among older dogs. Of 41 dogs below 6 months of age 63.4 per cent were positive, whereas 87.3 per cent of 71 dogs from 6 months to one year old were positive with titres in the dye test of 1:4 or higher.

This difference in infection rate between young and old dogs might be due to the fact that older dogs have had more time to acquire the infection and produce antibodies. A different explanation is offered by *Lainson* (1956) who emphasizes that young dogs are more susceptible to toxoplasma infection than older dogs. He suggests that because of this the likelihood of detecting toxoplasma antibodies among the lower age group is considerably diminished, since young infected animals usually die and are consequently not available for serological study. Whether this is correct must depend to a large extent also on the strain of toxoplasma involved.

In Table 5 the dogs are divided into two groups according to their geographical origin. 220 dogs came from outside the greater Copenhagen area and 734 dogs from within that area. It should be pointed out that this is not a distinction between urban and rural dogs. The difference between these two groups is merely geographical and the conditions under which the dogs are kept are probably fairly uniform. These facts are reflected in the figures which reveal no difference in the percentage of serologically positive individuals in the two groups.

Cats

It might have been expected that cats would be infected more frequently than dogs in the same area. The cat is more of a hunter than the dog and its diet is less well controlled. They may kill and eat a wide variety of birds, rats and mice. This is true not only of stray cats but also of family pets of which the present material is composed.

Of 184 cats 62.5 per cent were found to possess toxoplasma antibodies. Whether that percentage is representative for this area might be questioned. Firstly, the number of cats examined is much smaller than the number of dogs and secondly, all the cats included in this material were brought to the Small Animal Clinic for veterinary treatment which means that they are all family pets. No stray animals have been examined and, as already pointed out, one might expect an even higher percentage of serologically positive individuals in that group.

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Acta path microbiol scandinav 75 457–463 1969

The Virus Department of the Central Bacteriological Laboratory
of Stockholm City Sweden

AGE DISTRIBUTION OF ANTIBODIES TO EB VIRUS IN SWEDISH FEMALES AS STUDIED BY INDIRECT IMMUNOFLUORESCENCE ON BURKITT CELLS

By

ABERRA DEMISSIE and ARNE SVEDMYR

Received 15 viii 68

Herpes type virus particles referred to as the Epstein Barr or EB virus (EBV) have been observed in many continuous cell lines derived from Burkitt lymphomas lymph nodes or peripheral blood of patients with leukaemias or other diseases as well as from buffy coats of healthy donors (for references see 3 8) By immunofluorescence techniques a proportion of cells which contains EBV particles may be detected (2 3 5 9 20) Antibodies to the intracellular antigen(s) were found in high titre in all Burkitt tumour patients studied thus far (6) but have also been reported to be widely distributed in the general population (5 6 17) and to develop in association with mononucleosis in each of 29 patients studied (14)

The present report concerns the age distribution of such antibodies to EB virus in a Swedish population A direct comparison with the development of immunity to a common viral disease of childhood rubella will be made since the sera now investigated had previously been studied for rubella antibodies (12) Some of our results have been presented in a preliminary way (17)

Data concerning certain quantitative aspects as well as the reproducibility and specificity of the tests will also be presented

MATERIALS AND METHODS

S The 303 serum specimens investigated were collected in 1965 for a study of rubella immunity among females in the town of Eskilstuna (1) They were inactivated at 56 C for 30 min and stored at — 0 C

For control purposes sera from a few African patients with Burkitt lymphoma or glandular fever were employed as well as serum from a patient with embryonic a c m (Lemock Wilson) These specimens were kindly supplied by Dr *George Klein*

Requests for reprints should be addressed to Dr *Arne Svedmyr* Virus Department Central Bacteriological Laboratory P O Box 177 S-101 22 Stockholm Sweden

able indirect fluorescence However the incidence of stained cells varied widely (between 0 4 and 11 0 per cent) and like the more subjective impression of brilliance it was usually less than in the control

As seen from Table 1 there was no obvious difference between the sera of the different age groups as to variation in staining capacity all age groups contained sera that stained high as well as low proportions of cells

Thirty six positive sera representing the whole range of staining capacity were selected for further study such as end point titrations blocking tests and tests against other cell lines

End point titres varied between the extremes of 8 and 1024 and correlated roughly to the proportion of cells stained at the 1/8 dilution i e the dilution used in all screening tests (Table 2) Both the brilliance and the proportion of cells stained decreased with serum dilution When the percentage of cells stained with the serial dilutions were plotted for the individual sera a considerable variation in the slope of the curves was observed however This is illustrated in Fig 1 with the titrations of two of the Swedish sera and two specimens from African patients with Burkitt lymphoma all performed simultaneously under identical conditions

TABLE 2

End Point Titres of 36 Swedish Sera Compared with Proportion of Cells Stained at 1/8 Dilution

Percentage of cells indirectly stained with serum 1/8	Number of sera with respective titre							
	8	16	32	64	128	256	512	1024
9 0–10 5		–			–	1	5	2
6 9–8 5		–			–	4	4	
5 0–5 9		–			3	1	1	
2 5–4 0		–		2	2	1		
1 1–1 5	1	2	1	–	–			
0 6–0 9	–	2	3	–	–			

Reproducibility of Results

It should be noted that the results of indirect immunofluorescence tests were quite reproducible Thus the duplicate tests on the 36 selected sera the screening and the corresponding 1/8 dilution of the titration showed a variation in percentage of fluorescent cells that for individual sera amounted to one per cent or less The four titrations illustrated in Fig 1 had been carried out once before but on separate occasions While end point titres were identical for the same sera the proportions of fluorescent cells varied by at most 1 8 per cent for individual serum dilutions It might also be mentioned in this connection that six sera from patients with African glandular fever were titrated independently

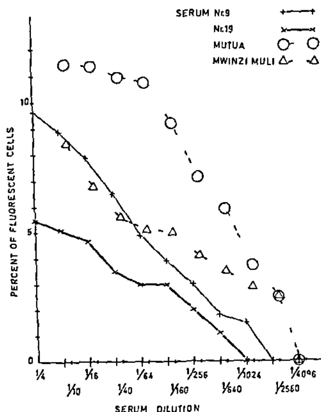


Fig 1

Proportion f Silfere cells indirectl stained by serial dilutions of four high titering sera

by Henle and Henle (personal communication) and by us results were identical three sera were negative at 1/10 and 1/8 respectively two had titres of 160 one 320

Blocking Experiments

When smears treated with a 1/8 dilut on of any of the 36 positive sera selected for further study w r ex osed o a 1/10 dilution of the unconjugated sheep antil uman g mr c bul preparation before the conjugated preparation was added r uorescence was seen

When sme rs were exp ed to selected sera (one serum was exhausted) or to the Mutua serum 1/100 prior to addition of the conjugated r direct immunofluorescence reaction was blocked althou varying degree apparently correlated to the staining capacity espective sera Thus 8 sera which at 1/8 dilution had stained 9 per nt or more of the Silfere cells blocked the staining completely when used diluted 1/8 8 sera staining between 6 9 and 8 per cent of s blocked completely when used

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Percentage of cells indirectly stained with serum 1/8	Number of sera with respective titres							
	8	16	32	64	128	256	512	1024
9.0–10.5	–				–	1		2
6.9–8.5	–		–			4	4	
5.0–5.9	–	–			3	1	1	
2.5–4.0	–	–		2	2	1		
1.1–1.5	1	2	–	–				
0.6–0.9	–	2	3	–				

Reproducibility of Results

It should be noted that the results of indirect immunofluorescence tests were quite reproducible Thus the duplicate tests on the 36 selected sera the screening and the corresponding 1/8 dilution of the titration showed a variation in percentage of fluorescent cells that for individual sera amounted to one per cent or less The *four titrations* illustrated in Fig 1 had been carried out once before but on separate occasions While end point titres were identical for the same sera the *proportions of* fluorescent cells varied by at most 1.8 per cent for individual serum dilutions It might also be mentioned in this connection *that six sera* from patients with African glandular fever were titrated independently

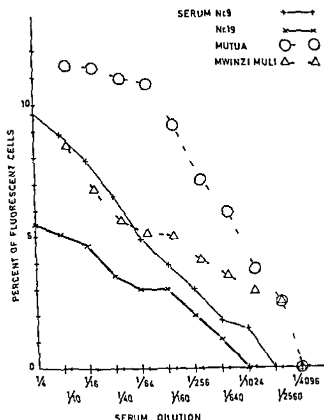


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Blocking Experiments

When smears treated with a 1/8 dilution of any of the 36 positive sera selected for further study were exposed to a 1/10 dilution of the unconjugated sheep antihuman gammaglobulin preparation before the conjugated preparation was added no fluorescence was seen

When smears were exposed to any of 35 selected sera (one serum was exhausted) or to the unconjugated Mutua serum 1/100 prior to addition of the conjugated Mutua serum the direct immunofluorescence reaction was blocked although to a varying degree apparently correlated to the staining capacity of the respective sera Thus 8 sera which at 1/8 dilution had stained 9 0 per cent or more of the Silfere cells blocked the staining completely when used diluted 1/8 8 sera staining between 6 9 and 8 5 per cent of cells blocked completely when used

TABLE 3

Titration of Blocking Capacity of a Swedish Serum and the Standard Mutua Serum

Serum used for blocking	Per cent cells stained by Mutua conjugate after exposition to respective serum dilution									
	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Swedish serum no 9	0	0	2	3 7	4 1	5 2	5 7	–	–	–
Mutua 2-11-67	0	0	0	0	2 5	4 0	4 9	5 0	5 0	5 7

Control stained directly with the conjugated Mutua preparation 6 1 per cent

undiluted but only partially at 1/8 19 sera staining between 0 6 and 3 9 per cent blocked only partially even when used undiluted and to a somewhat unpredictable extent between 0 2 and 1 6 per cent still being stainable by the conjugated Mutua serum Without prior exposition to other sera 6 5 per cent were stained Six undiluted sera from 2 year old children which were completely negative in the screening test at 1/8 dilution did not even partially block the conjugated Mutua serum (5 9–6 5 per cent of the cells stained)

The blocking capacity seemed to be better correlated to the proportion of cells indirectly stainable with serum diluted 1/8 than to the end point titre This discrepancy might be expected since as exemplified above (Fig 1) sera with the same end point titre may at low dilution elicit fluorescence in quite different proportions of cells

Table 3 illustrates the gradual reduction of the blocking capacity on serial dilution of two strongly positive sera the Swedish serum nr 9 and the standard Mutua serum (end point titres 1024 and 2560 respectively cf Fig 1) Complete blocking was obtained with the sera diluted 1/16 and 1/64 respectively

Tests with Cell Lines other than Silfere

The 36 selected sera diluted 1/8 were also tested by the indirect method against another Burkitt cell line *Esther* *The proportions of fluorescent cells obtained with this cell line were similar to the corresponding values obtained with Silfere cells* whether the Mutua control *serum or the* Swedish sera were used A maximal variation of 2 3 *per cent* between the results obtained with the two cell lines was observed

As a further control 20 representative sera out of the group mentioned above were tested in the same manner against the cell lines *SKL 1* and SKL 2 These are reported to contain a very low frequency *of EBV* positive cells (5 11) No fluorescence was observed neither with *the* Mutua control serum *diluted 1/100 nor* with any of the Swedish sera

DISCUSSION

It seems to be firmly established by now that antigen(s) associated with EB virus infection are specifically stained by immunofluorescence techniques similar to those used in the present study (2 3 20) Our results indicate therefore that EB virus or other agents closely related to it are widely disseminated in the Swedish population The age distribution of antibodies in Swedish as well as in American (6) population groups shows that EBV infections occur at a similar rate as the common viral diseases of childhood as suggested by the present study the EBV infection may actually be more contagious than rubella It may be pointed out that contagiousness of the human infection does not necessarily mean that a virus is easily transferred between tissue cultures the discrepancy between the contagiousness of chickenpox and the difficulty to obtain free infectious varicella virus in most tissue culture systems is a wellknown example concerning a herpesvirus (16 21)

The Henles et al (22 14) recently concluded that EB virus or a closely related one is probably aetiologically related to mononucleosis This clinical syndrome is however diagnosed most often in young adults Niederman et al (14) suggested therefore as did Evans previously on epidemiological grounds (4) that the infections in children are seldom diagnosed as mononucleosis possibly since they may rarely develop the typical clinical haematological and serological features Further studies of the epidemiology of EBV infections and the possible relationship to mononucleosis and other clinical syndromes are obviously indicated particularly in children Whether the EB virus so often found in Burkitt cell lines is aetiologically related to the tumour disease or is merely a passenger virus remains another intriguing question

EB virus or antigenically similar agents seem to have a worldwide distribution (5 6 17) The results of the blocking tests reported above indicate a close antigenic relationship between the agent(s) prevalent among African Burkitt patients and that (those) widely disseminated in the Swedish population Whether the viruses are identical or only antigenically related must be left an open question however It may be mentioned in this connection that immunological cross or recall reactions have been observed in convalescent sera between such distinctly separate members of the herpesvirus group as the herpes simplex and the varicella viruses (10 15 16) Similar cross reactions have so far not been seen between the EB virus and other herpesviruses (3 5 7)

Although the general pattern of immunization against EB virus seems evident the reactivity of sera which are positive in the indirect immunofluorescence test varies widely Antibody concentrations apparently vary within wide limits in all age groups also in the recent American study (6) and the results do not suggest any correlation between antibody concentration and the age of the person similar to that observed in the corresponding investigation on rubella immunity (12) The observation that some high tilering sera at low dilution

stained much fewer cells than did others of the same titre may suggest that several antigens are involved the concentrations of which vary independantly in different cells and that some individuals do not develop antibodies to a similar extent towards all the antigens It should be noted that other herpesviruses like herpes simplex virus (19) and varicella virus (18) are known to give rise to several distinct antigens

SUMMARY

In a Swedish population of 307 females the age distribution of antibodies to EB virus was investigated by the indirect immunofluorescence technique with acetone fixed EBV positive cells of Burkitt origin The incidence of reactive sera was about 90 per cent at birth dropped to 12 per cent at 2 years of age and then rose steeply to reach again 90 per cent at 20 Antibodies against EB virus or antigenically related agents were acquired somewhat earlier than neutralizing antibodies against rubellavirus The reactivity of positive Swedish sera measured by the proportion of cells stained with a low serum dilution as well as by end point titrations varied within wide limits but was in most cases considerably lower than that of the positive control serum which originated from a Burkitt patient While end point titres correlated roughly to the proportions of cells stained at low serum dilution some high titering sera at low dilutions stained much fewer cells than did others of similar titre Swedish sera with high anti EBV reactivity completely blocked the direct staining with a conjugated serum preparation from an African Burkitt patient

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Acta path microbiol scandinav 75 466–480 1969

The Department of Immunochemistry The Wallenberg Laboratory
University of Uppsala Uppsala Sweden

PROTEIN A FROM *STAPHYLOCOCCUS AUREUS*

VII Physicochemical and Immunological Characterization

By

ARNE FORSGREN and JOHN SJÖQUIST

Received 22 viii 68

Protein A was originally characterized as a cell wall constituent of most *Staphylococcus aureus* strains which precipitates human γ globulin (*Jensen* 1958) It was later shown that protein A also precipitates normal rabbit (*Forsgren & Sjöquist* 1967) and guinea pig γ globulins (*Lofkvist* 1966 *Forsgren* 1968 A) The protein has been found to interact with the Fc fragment of the γ globulin molecules (*Forsgren & Sjöquist* 1966 1967 and *Forsgren* 1968 B)

The protein A used in some of the experiments reported (*Forsgren & Sjöquist* 1966 1967 and *Forsgren* 1968 A B) was obtained after extraction of the bacteria in phosphate buffer pH 5 9 at 100 C The protein was precipitated from the extract at pH 3 chromatographed on DEAE Sephadex and gel filtered on Sephadex G 100 A similar preparation was characterized by *Grov et al* (1964) and *Grov* (1965 1967) and found by them to be a homogeneous product with a molecular weight of 12 000 The material had one N terminal amino acid alanine and was composed of only a few amino acids The data for our preparation do not agree with those of *Grov et al* (1964) and *Grov* (1965 1967) On the contrary the preparation seems to be very heterogeneous as judged from 1) N terminal amino acid determinations 2) gel filtration and 3) electrophoresis in polyacrylamide gel

MATERIALS AND METHODS

Strain and Cultivation

S aureus serologic type Cowan 1 was used The bacteria were grown in a 500 l tank at the pilot plant of the Department of Bacteriology Karolinska Institutet

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We are indebted to Drs *H Pertoft* and *A Lundblad* Institution of Medical Chemistry Uppsala and to Dr *D Eaker* Institution of Biochemistry Uppsala for aid with analytical ultracentrifugation carbohydrate determination and amino acid analysis respectively The excellent technical assistance of Mrs *I Möller* and Mrs *I Sjöquist* is gratefully acknowledged

This work was supported by grants from *the Swedish Cancer Society* (Project No 56 B67 02X) *the Swedish Medical Research Council* (Project No K69 13X 6 04B) and *Knut och Alice Wallenbergs Stiftelse* One of the authors (A F) was the recipient of a predoctoral fellowship from the University of Uppsala

Stockholm Sweden The culture medium consisted of th following in the proportions indicated 1 l tap water 5 g meat extract 3 g NaCl 2 g NaH PO ?H O 1 g glu o e 10 g peptone pH was 7 8 After 14 hrs incubation at 37° C the bacteria were harvested by centrifugation and washed twice in saline

Preparation of Crude Protein A

Heat extraction was performed as des ribed by *Jensen* (1959) in phosphate buffer (9 parts 1/15 M KH PO_4 + 1 part 1/15 M Na HPO pH 5 9) in a boiling water bath for 1 hour followed by rapid cooling to 4 C The transparent yellow supernatant was adju ted to pH 3 0 with 0 1 N HCl and the precipitate formed was collected by centrifugation and dissolved in phosphate buffer pH 5 9 Ethanol was added to the clear solution to a final concentration of 70 per cent and the resultant pre ipitate was spun down and dissolved in phosphate buffer pH 5 9 Finally trichloroacetic acid was added to a concentration of ? per cent and the resultant precipitate was collected dissolved in phosphate buffer pH 9 dialyzed against distilled water at +4 C for ?–3 days and lyophilized In some experiments the ethanol and trichloro acetic acid precipitation steps were omitted

Separation Methods

Ion Exchange chromatography Crude preparations of protein A obtained by heat extra tion were taken up in 0 1 M NH_4HCO_3 and applied to a DEAE Sephadex column (Pharmacia Fine Chemicals AB Uppsala Sweden) equilibrated with the same buffer Elution v as started with 0 1 M NH HCO_3 When the first peak of UV absorbing material had merged from the column the elution buffer was changed to 0 4 M NH HCO_3 A second UV absorbing peak was colle ted and each fraction was assayed for protein A A f w fractions in the beginning of the second peak contained most of the protein A active material (90–95 per cent) They were combined dia lyzed against distilled water and lyophilized

Gel filtration Protein A preparations purified on DEAE Sephadex were chromatographed on a column of Sephad x G–150 (95 × ?5 cm) in 0 05 M Tris HCl 1 M NaCl (*Flodin & Killander* 196?) or in some experiments on a column of Sephadex G–100 Less than ?00 mg mat rial was applied to the column Collected fractions (7 5 ml) were tested for protein A activity

Isoele tric focusing Heat extracted protein A purified by DEAE Sephadex chromatography was separated by isoelectric focusing as described by *Svenson* (196?) and *Vesterberg & Svenson* (1966) An electrofocusing column (440 ml) equipped with a cooling mantle and platinum electrodes was used (LKB Sto kholm Sweden) Synthetic ampholytes (poly amino poly carboxylic acids LKB) with isoelectric points bet veen 3 and 10 were used to estimate the apparent pI of the protein A active material A 1 per cent ampholyte solution giving a pH gradient was made up ac cording to *Vesterberg & Svenson* (1966) To prevent precipitation of proteins in the column 6 M urea was incorporated (*Jeppson* 1967) Urea was crystallized from a warm ethanol solution which had been passed through a mixed ion exchange resin During the runs the columns were kept at 4 C by a cooling bath Hetofrig CC (Heto Birkerod Denmark) DEAE Sephadex purified protein A (100 mg in 9 ml) was applied in the middle of the gradient The eluate was collected in 4 ml fractions The pH of each fraction was measured with a Radiometer pH meter model 2, and a Radiometer glass electrode type GK ?0?1 C. Each fraction was dialyzed against 0 05 M Tris HCl pH 8 0 and tested for protein A activity and protein con tent (*Lowry et al* 19 1)

Electrofo using v as run for 24 or 48 hours The initial conditions were ?00 V 2 5 mA The voltage was successively increased during the run to a final value of 400 o 800 V

Isolat on of p otein A from human γG-globul n—protein A precipitates was performed essentially as described by *G (et al* (19?4) Heat extracted DEAE Sephadex purified protein A (50 mg in 5 ml) and human γG globulin (?30 mg in 10 ml) in ph phate buff red saline pH 7 –7 3 were mixed and incubated at 37 C for 2 h urs The precipitate was colle t d by centrifugation wa hed twice with cold saline and dis ol ed in 5–8 ml of 0 0 N HCl–0 15 M NaCl (*Grol et al* 19 4) The sample was then applied to a column f Sephadex G 100 (85 × 3.5 cm) which was quilib ated and eluted with the HCl N Cl solution Elution v as performed at 4 C and 15 20 ml hour Absorbancies of the fractions at 280 mμ were read in a ...

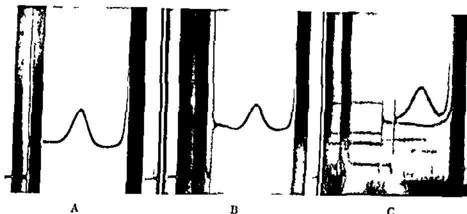


Fig 1

A and B Schlieren patterns for velocity experiments on DEAE Sephadex purified protein A (a) and the same material further separated by preparative polyacrylamide electrophoresis (b) in a synthetic boundary cell at a rotor speed of 59 780 rpm 65 min after reaching full speed a) protein concentration = 10 mg/ml bar angle = 60 S_{0w} = 1 42 b) protein concentration = 8 mg/ml bar angle = 50 S_{0w} = 1 70—*C* Schlieren pattern for an approach to sedimentation equilibrium experiment (Archibald) on protein A separated from human γG globulin protein A precipitates in a double sector cell at a rotor speed of 42 040 rpm 48 min after reaching full speed The protein concentration was 10 mg/ml bar angle 60 and S_{0w} = 1 81

RESULTS

Ion Exchange Chromatography and Gel Filtration

Chromatography of the crude preparations of protein A on DEAE Sephadex gave an 8–10 fold purification This material formed a homogeneous peak in ultracentrifugation (Fig 1A) The sedimentation constant (S_{0w}) at a concentration of 10 mg/ml was 1 42 However analytical electrophoresis in polyacrylamide gel (Fig 2A) showed distribution of the material in a broad band within which a few more distinct bands were seen Anodic to the broad band 1–3 faint lines were visible The protein A activity was shown to be associated with the broad band by diffusion in agar gel against human γG globulin

Protein A material from DEAE chromatography was chromatographed on Sephadex G 100 or G 150 in 0 05 M Tris 1 M NaCl pH 8 0 The active material was eluted from the G 150 column (80 × 2 5 cm) in a single broad peak between 210–420 ml The protein A activity was distributed over the whole peak with specific activity slightly higher in the front fractions and significantly lower in the last 10 per cent of eluted material Part of the last fractions corresponded to the faint lines seen in analytical polyacrylamide gel electrophoresis (Fig 2A)

Preparative Polyacrylamide Electrophoresis

Fig 3 shows the elution curve of material purified on DEAE Sephadex when subjected to preparative electrophoresis in polyacrylamide

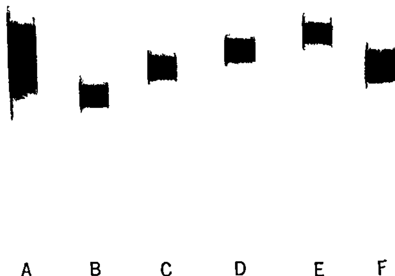


Fig 2

Analytical polyacrylamide electrophoresis at pH 9.5 of: A, DEAE Sephadex purified protein A.—B–F, Fractions (22–23, 32–33, 42–43 and 52–53 respectively) from the preparative polyacrylamide electrophoresis shown in Fig 3.—F, Pooled protein A after preparative polyacrylamide electrophoresis. The samples (0.2 mg) were applied to each gel in a volume of 0.1 ml and the electrophoresis was run for 30 min. The bottom of the photograph is anodal.

gel. The two curves represent the absorbancy at 280 mμ and at 540 mμ (Lowry reaction) respectively. Protein A activity, which was demonstrated in fractions 17–58 (dotted line), seemed to be associated with the Lowry positive material. Four fractions containing material from fractions 22–23, 32–33, 42–43 and 52–53, respectively, were dialyzed separately against distilled water, lyophilized and analyzed by electrophoresis in polyacrylamide gel (Fig 2B–E). As shown in the figure the four fractions had significantly different mobilities. Each fraction gave a precipitate in agar gel against normal human γG globulin corresponding to the position of its band in the polyacrylamide gel. The fractions thus represent materials with protein A activity but of different composition. The specific activities of the fractions were approximately the same. The marked asymmetry of the elution curve (Fig 3) may be partly due to the drop in current towards the end of the run.

Material corresponding to fractions 20–38 in Fig 3 was combined and re-electrophoresed. The most active fractions from the second run were combined, dialyzed against distilled water and lyophilized. The electrophoretic mobility of this preparation is shown in Fig 2F. The spe

recovered. Its specific activity was approximately the same as that of the starting material. Electrophoresis for a longer time and at a higher voltage gave a similar pH distribution of active material. However, under these conditions there was a considerable loss of activity.

Dissociation of Protein A from Human γG globulin

Thoroughly washed precipitates obtained by mixing human γG globulin and crude or DEAE-Sephadex purified protein A preparations were dissolved in 0.02 M HCl—0.15 M NaCl (*Givol et al.* 1962). The components were then separated on a Sephadex G-100 column equilibrated and eluted with the same solvent. Protein A active material was recovered in fractions with low absorbancies at 280 mμ that emerged from the column after the elution of a large protein peak with the void volume. The active fractions were combined, dialyzed against distilled water and lyophilized. The recovery of active protein A was 15–20 per cent. In later experiments the recovery could be increased to 40–50 per cent by using Sephadex G-200 in 2 M formic acid. Addition of 6 M urea to the dissociating and eluting solvent did not increase the recovery.

In ultracentrifugation the recovered material (10 mg/ml) gave a symmetric peak (Fig. 1C) with a calculated $S_{20,w}$ value of 1.81. Molecular weight determinations (*Archibald* 1947, *Ehrenberg* 1957 and *Trautman & Crampton* 1959) gave a value of 31,700. This is approximately twice the molecular weight of the material obtained from preparative polyacrylamide electrophoresis. It is possible that a polymerization of protein A occurs during reaction with human γG globulin. The low recovery of protein A may be due to incomplete dissociation or to polymerization or to a combination of both events. The specific activity of the dissociated material and its electrophoretic properties in polyacrylamide were approximately the same as those of the DEAE-Sephadex purified preparation.

Amino Acid Analyses

Table 1 presents the analyses of three different preparations of protein A: 1) crude material purified by DEAE chromatography, 2) this material further electrophoresed twice on polyacrylamide, and 3) protein A obtained by acid dissociation from a precipitate with γG globulin. Except for tryptophan and ½ cystine all the common amino acids were found. The only obvious difference between the preparations is in aspartic acid. The amino acids account for 87.5–92.5 per cent of the weight corrected for ash and moisture.

Amino acid analyses were also performed on several other preparations. For example, the DEAE-Sephadex chromatographed preparation was further separated by gel filtration on Sephadex G-100 followed by isoelectric focusing. The amino acid composition of the active material showed only minor deviations from that presented in Table 1. Similar

TABLE 1

Amino Acid Analysis of Heat Extracted Protein A Further Purified as Indicated

Amino acid	Purified by DEAE Sephadex chromatography		Purified by DEAE Sephadex chromatography + polyacrylamide electrophoresis		Purified by dissociation from human γG globulin	
	µmol/g	g/100 g protein	µmol/g§	g/100 g protein	µmol/g§	g/100 g protein
Try	–	–	–	–	–	–
Lys	674 9	11 0	740 8	11 9	8?3 2	1? 8
His	86 4	1 5	77 0	1 3	68 1	1 1
Arg	180 1	3 6	149 9	2 9	138 6	2 ?
Asp	1183 1	17 2	13?3 7	14 4	1400 0	19 7
Thr	283 4	3 7	??4 1	2 9	207 3	2 6
Ser	40? 9	4 5	499 4	5 4	411 6	4 4
Glu	1207 5	19 7	1336 9	?1 9	134? 1	21 ?
Pro	240 1	3 0	312 0	3 9	3?4 6	3 9
Gly	375 5	? 7	403 7	2 9	38? 5	2 7
Ala	606 1	5 4	689 1	6 1	658 0	5 7
½-Cys	–	–	–	–	–	–
Val	380 0	4 8	284 6	3 6	269 9	3 3
Met	120 5	? 0	83 3	1 4	76 2	1 2
Ile	357 0	5 2	317 7	4 5	273 1	3 8
Leu	545 7	7 8	6 8 4	8 8	553 6	7 7
Tyr	147 6	3 0	133 6	? 7	12? 0	2 4
Phe	269 4	4 9	298 2	5 5	?78 3	5 0

Linear extrapolation to zero hours

§ The figures are not corrected for ash and moisture

results were obtained if the DEAE Sephadex chromatographed material was run through a Sephadex G 150 column and individual fractions of the active peak were analyzed

N terminal Amino Acids

Table 2 gives the results of N terminal determinations on the preparations presented in Table 1 Although alanine is the main N terminal amino acid 4–6 additional N terminals were found and it may be concluded that all three preparations represent heterogeneous material The preparations were also analyzed for N terminal arginine and histidine which however were not found

*Three individual fractions from the isoelectric focusing of DEAE Se*phadex chromatographed material with apparent pI s of 5 06 5 11 and 5 20 were analyzed Each fraction within the active *peak represented* 0 01 pH unit These fractions showed the same heterogeneity as did the materials presented in Table 2 although the recovery of each N terminal varied among the fractions Similar results were obtained when individual fractions from a Sephadex G 150 chromatography were analyzed

parts of the molecules In contrast to what has been reported (*Grov* 1967) it is impossible to state from the present data that protein A is built up of a single polypeptide chain

That the heterogeneity of *protein A is not due only to the initial ex*traction step is shown by analyses of highly purified material obtained by different extractive techniques Lysozyme digestion of *S aureus* cells *releases a considerable amount of* protein A active material (*Forsgren* 1968 C) Purification of this material by ion exchange chromatography gel filtration and preparative polyacrylamide electrophoresis yielded a product that showed the same degree of heterogeneity as the purified product of heat extraction (*Forsgren & Sjoquist,* unpublished data) Furthermore gel filtration of a product obtained by initial disintegration of the cells at —20 C showed the material to be distributed over a wide *range suggesting its heterogeneity* (*Lofkvist & Sjoquist 1963*)

Thus in all our experiments to date protein A has proved to be heterogeneous by chemical criteria This does not necessarily means that *protein A is not a homogeneous material biologically All subfractions of* the purest products showed almost the same specific activity when tested against human γG globulin The observed heterogeneity of protein *A may be the result of the extraction and purification* techniques used or may be a true heterogeneity due to the fact that different mutant cells synthesize protein A in different forms In any case further studies are necessary to establish the true nature of protein A

SUMMARY

Heat extracted protein A from *S aureus* purified by DEAE Sephadex chromatography has been further separated and characterized physico chemically By gel filtration electrophoresis in polyacrylamide gel and isoelectric focusing *protein* A has been found to be heterogeneous Fractions with different properties in the separation systems proved to have approximately the same specific activity against human γG globulin in agar gel

Protein A purified by chromatography on DEAE Sephadex and electrophoresis in polyacrylamide gels gave a symmetrical peak on ultra *centrifugation* with a $S°_{20,w}$ of 1 6 and a calculated molecular weight of 15 000 Protein A dissociated from human γG globulin prot*ein A pre*cipitates gave a molecular weight of 32 000 and was supposed to be a dimeric form

Amino acid analysis of various protein A preparations obtained by different separation methods showed approximately the same composition The amino acids found amounted to about 90 *per cent of the dry* weight All the common amino acids except ½ cystine and tryptophan were found Five to seven N terminal amino acids were *found in all pre*parations studied although variations in the *quantitative distribution of the* N *terminals* among the preparations were found

Protein A purified by chromatography on DEAE Sephadex and by electrophoresis in polyacrylamide gel gave two precipitation lines against rabbit anti protein A γG globulin in agar gel diffusion After pepsin digestion of the γG globulin only one line was seen indicating immunological purity of the protein A preparation

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The Department of Immunochemistry The Wallenberg Laboratory
University of Uppsala Uppsala Sweden

PROTEIN A FROM *STAPHYLOCOCCUS AUREUS*

VIII Production of Protein A by Bacterial and L Forms of S aureus

By

ARNE FORSGREN

Received 29 viii 68

The cell wall of *Staphylococcus aureus* contains two major components ribitol teichoic acid and mucopeptide which have been reported to together comprise 66–91 per cent of the cell wall (*Rogers* 1962 *Rogers & Perkins* 1968) Protein A has been reported to be an additional constituent of the *S aureus* cell wall (*Yoshida et al* 1963 *Grov & Rude* 1967 A B) It has been purified from intact cells and from cell wall preparations and has not been detected in the culture medium (*Jensen* 1958 1959)

Protein A was originally described as a substance precipitating human γ globulin and responsible for the agglutination of *S aureus* by normal human sera (*Jensen* 1958 1959) Later it was shown that protein A also reacts with normal guinea pig (*Lofkvist* 1966 *Forsgren* 1968 A) and rabbit γ globulins (*Forsgren & Sjoquist* 1967 1968) Protein A has been found to interact with the Fc fragment of non immune γG globulin (*Forsgren & Sjoquist* 1966 1967 and *Forsgren* 1968)

The purpose of this investigation was to determine whether protein A is produced by cells lacking cell walls (L-forms) and whether it can be detected in the culture media of coccal and L-forms

MATERIALS AND METHODS

Strain A *Staphylococcus aureu* strain t pe Cowan I (NCTC 8530) kindly pr vid b Dr A Cro and Profe sor I Oeding Bergen N rwa was used thr ughout t investigation The strain has a high c nt nt l protein A (*J nsen* 1959)

Media and cultivation of S aur us The ba er al f rm of S *aureus* Cowan I grown in Nutrient Broth (Difco) Aeration hi ved l shaking in Erle r flasks on a platform sl ker G r wth e l turb limetrically in l l tt Summers n olorimeter equipped ith f (Spectral range 640– 00 mμ The bacteria vere harvested by centrif e t 0 × g for 20 min Bef r th broth

The ex llent technical assi tan / M ller is gratefully k owl dged
This work was supported by gra r th Swedish Cancer S ty (Project No 56 B67 0 X the Swedish Med Council (Project N B 8 13X 620 04B) and Knut h Al Wallenb rg The autho was th r cipient of a predoct ral f ll wship from the Unive t f U p al

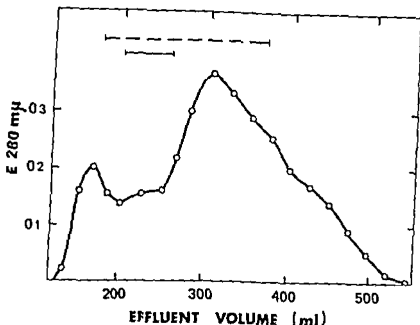


Fig 1

Gel filtration in 0 05 M Tris HCl 1 M NaCl pH 8 0 on Sephadex G 1?0 (? 5 by 9? cm) of 180 mg protein A obtained by incubation of *S aureus* with lysozyme in 0 0? M Tris HAc pH 8 0 and purified by chromatography on DEAE Sephadex Protein A detectable in agar gel immunodiffusion against 1 per cent normal human γG globulin in undiluted fractions and in fractions diluted 4 fold is indicated by ⊢ – – and ⊢—— respectively

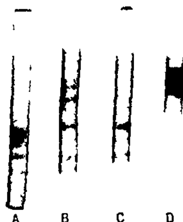


Fig 2

Analytical polyacrylamide electrophoresis at pH 9 ? of different stages of purification of protein A obtained by lysozyme digestion A) The supernatant of the digestion mixture after dialysis B) After chromatography on DEAE Sephadex C) After gel filtration on Sephadex G 150 and D) After preparative polyacrylamide electrophoresis The sample (0 ? mg) was applied to each gel in a volume of 0 1 ml and the electrophoresis was run for 60 min The bottom of the figure is anodal

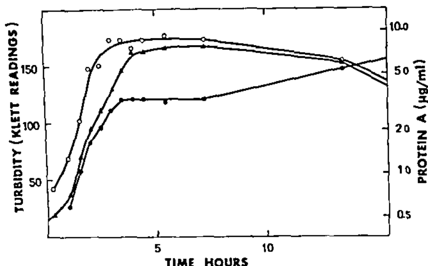


Fig 3

Growth curve for *S aureus* Cowan 1 (▲—▲—▲) Protein A detected in cells (○—○—○) and in broth (●—●—●) is also indicated The amount of protein A in cells and in broth was determined with reference to standard curves made up for purified heat extracted and lysozyme extracted protein A preparations respectively

Digestion with Lysozyme in the Absence of Sucrose

Lysozyme digestion in 0 02 M Tris HAc pH 8 0 for 24 hrs at 25 released more protein A than did digestion in buffered sucrose In the absence of sucrose the turbidity decreased to 20–40 per cent Protein A was purified from the dialyzed supernatant from digested cells by chromatography on DEAE Sephadex gel filtration on Sephadex G 150 (Fig 1) and preparative electrophoresis in polyacrylamide

These three purification steps gave a 12 fold increase in specific activity with an over all recovery of 50 per cent Analytical polyacrylamide electrophoresis of fractions of different degrees of purity are shown in Fig 2

Protein A obtained in this way gave the same immunological reactions with γ globulins from different species as did heat extracted protein A (*Forsgren & Sjoquist* 1966 1967 1968 and *Forsgren* 1968 A B) However the precipitation lines in agar gel were more distinct and the specific activity was 2 3–2 8 times higher as determined by the single radial immunodiffusion technique

Protein A in S aureus Cells and in their Medium

Fig 3 shows a growth curve for *S aureus* in nutrient broth registered by measuring turbidity in a Klett Summerson spectrophotometer The protein A content of the bacteria and the broth is also indicated in the

digested cells indicating that protein A is mainly a surface component of the bacteria

Protein A released by incubation with lysozyme and purified by several techniques has a higher specific activity than does heat extracted protein A when assayed in agar gel immunodiffusion against human γG globulin. The lower activity of heat extracted protein A is probably due to loss of activity during extraction. The protein obtained by lysozyme digestion was supposed to be more like the protein A released during growth and was therefore used as a standard preparation for the quantitation of released protein A.

By immunological methods protein A can be detected in the culture medium of *S aureus*, Cowan 1 as well as in the cells. About thirty per cent of the total protein A produced can be detected extracellularly during the logarithmic growth phase and in the phase of decelerated growth. This is in contrast to the results presented by *Jensen* (1958, 1959) who could detect no protein A in the broth after 12, 18 or 24 hours. However Jensen does not mention concentrating the broth and we also failed to detect protein A in the culture medium without concentration. After incubation of a concentrated cell suspension (10^{10} bacteria/ml) for some days *Jensen* (1959) observed significant amounts of protein A in the unconcentrated incubation fluid.

Cell wall constituents of various bacteria have been shown to be absent or decreased in amount in the respective L forms (*Morrison & Weibull* 1962, *Sharp* 1963). The absence of cell wall material in L-forms of *S aureus* suggested by the absence of chemically and immunologically detectable mucopeptide and cell wall teichoic acid has recently been reported (*Sharp* 1963, *Pratt* 1966). Osmotic fragility and insusceptibility to phage action are further indications that the surface structure of the L forms is altered (*Williams* 1963). However immunological identity between cytoplasmic and membrane material from bacterial and from L forms of *S aureus* has been reported (*Pratt* 1966). The L-forms resemble their parent cocci in the qualitative production of many enzymes such as catalase, coagulase, deoxyribonuclease, gelatinase and lipase (*Smith & Willis* 1967).

The results reported here show that protein A, which has been prepared from cell wall preparations (*Yoshida et al* 1963) and reported to be a cell wall constituent, is produced in L-forms. Protein A can be detected both in the L-forms and in the L-form broth but in smaller amounts than in the bacterial form. Antigenic cell wall substances have previously not been found in four strains of L forms of *S aureus* examined (*Pratt* 1966). As protein A is a group antigen specific for and present in nearly all strains of *S aureus* (*Oeding & Haukenes* 1963) its presence in L-forms provides an additional criterion for their identification.

We have found that protein A can be liberated from *S aureus* without lysis and that it is released into the medium even during the logarith

mic phase of bacterial growth The release of protein A can be an important factor in the pathogenecity of *S aureus* as it has been shown that the interaction between protein A and γG globulin elicits hyper sensitivity reactions (*Gustafson et al* 1967 1968 and *Sjoquist et al* 1967) Even L forms have been found to produce a significant amount of protein A

SUMMARY

Incubation with lysozyme releases most of the protein A from *S aureus* without lysing the bacteria The protein A released by lysozyme diges tion was purified by chromatography on DEAE Sephadex gel filtration on Sephadex G 150 and preparative polyacrylamide electrophoresis This preparation was used as a reference for quantitation of the protein A released during growth of *S aureus* and its corresponding L-form

It was found by immunological techniques that about thirty per cent of the protein A produced by the bacteria is released into the broth during the logarithmic growth phase

Protein A was detected in the L-forms of *S aureus* as well as in their culture medium The concentration was approximately 20 times lower in the L forms than in the corresponding bacterial forms

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Jensen K A normally occurring staph l i antibo l in human serum A ta path microbiol scandinav *44* 4 1 4 9 8
Jensen K U ndersogelser over st ph l nt genstrul tur Med sp ielt hen blik på et polysacchar danti lt for kommende antist fr r imod dett D p (Munksgaard I I n a l)
Lofkvist T Preparat ons of purif n from *Staph ylococcus aur us* in bio logi and serol gic experiment f f f fract ons A 1 A A 3 B and C n dermal re ctivity of the r i n phrine and on isolat d ileum from normal guinea pigs O curren pitins n sera from normal rabl t and guinea pigs Int Arch All r 19 – 07 196

For analysis of the K88 antigen use was made of culture and/or antiserum corresponding to the following strains:

C1953 = O147 K89(B) K88ac(L) H19 (test strain of K88ac)
C7 = O8 K87(B) K88ab(L) H19
E68 = O141 K85(B) K88ab(L) H4 test strain of K88ab)
Bi623-42 = O11 K10 H10 (test strain of H10)

Preparation of Antisera, Antigen/Antibody Reactions and Absorptions

Antisera were prepared in rabbits: broth culture heated at 100 C for 2 hours was employed for O antiserum production and formalinized suspension of broth agar culture in the case of OK antiserum. For details see *Kauffman* (4) and *Ørskov et al* (11).

Media

Ox heart broth infusion was used for plates (1.6 per cent agar) with or without the addition of 0.1 per cent fluid Pril® (Henkel) and beef broth for fluid medium. In both cases 1 per cent peptone, 0.3 per cent NaCl and 0.2 per cent $Na_2HPO_4 \cdot 12H_2O$ were added. For detection of K88 forms modified Drigalski plates (4) with 1 per cent lactose and bromothymol blue as indicator were employed.

Treatment with Acridine

Tubes containing 5 ml of broth (pH 7.6) with varying concentrations of acriflavine (2) were inoculated with 0.1 ml of an overnight broth culture diluted to 10^{-3}. After 24 hours at 37 C samples were plated from the first tube with visible growth and from the control tube on to bromothymol blue plates.

Staining of Flagella

Leifson's method was used (5).

RESULTS

The strains A1 and CS1483 were found to be serologically identical on the basis of cross absorption tests performed with both O and OK antisera. The following account deals with the serological behaviour of CS1483 only, since this strain, the first of a series of antigenically similar strains from D.D.R., was received in 1963.

In the O antigen analysis CS1483 was found to be related to O1, O112ac, O117 and O144. Cross absorption tests showed that the O antigen of CS1483 was not identical with any of these factors and it was therefore given a new number, viz. O149.

According to the H antigen determination the H antigen of CS1483 was assigned to H10.

The presence of antigen K88 was demonstrated through the agglutinability of unheated culture in test K88ab and K88ac antisera. Before the result of the investigation of K88 antigen factors is presented the question of the presence of yet another K antigen will be dealt with. In all cases hitherto examined the presence in K88 strains of an additional K antigen of the B type has been revealed by the agglutination characteristics of unheated culture grown at 18 C:

1) Failure to agglutinate in a heterologous K88 antiserum, i.e. antiserum prepared against a strain also possessing the K88 antigen but

having no other antigens in common with the strain under investigation This means that no K88 antigen is developed at 18 C

2) No reaction in specific O antiserum the so called O inagglutinability

3) Ability to agglutinate in homologous OK antiserum indicating the presence of a K antigen provided the culture is O inagglutinable

4) Failure to react in homologous OK antiserum absorbed with boiled homologous culture By this procedure the K(B) antibodies are removed (A positive reaction in this serum is indicative of the presence of another K antigen but of the L type which in contrast to K88 is developed at 18 C)

In Table 1 the reactions are presented schematically and compared with those obtained with culture grown at 37 C

TABLE 1

Agglutination Reactions of K88 Antigen Cultures Grown at 37 C and 18 C

Culture	Sera			
	Homologous OK		O	Heterologous K88
	unabs	abs with homol 100 C		
37 C	+	+		+
18 C	+	−(B) +(L)		—

Instead of cultivating the strain at 18 C a variant without the K88 antigen grown at 37 C may be employed As colonies representing the K88 form of CS1483 did not appear spontaneously a K88 form was isolated by means of acriflavine known to be able to cure for the K88 antigen

However it can be seen from Table 2 that irrespective of cultivation temperature and presence or absence of K88 plate cultures of CS1483 agglutinated to a fairly high titre in OK antiserum CS1483 absorbed both with boiled culture of CS1483 and motile H10 culture The agglutination was interpreted as H agglutination in view of the physical characteristics of the reactions the demonstration of flagella in stained preparations and the fact that high titres were obtained in parallel test using test H10 antiserum In accordance with this interpretation the H antigen of CS1483 was considered not to be identical with H10 The OK antiserum CS1483 could not be [illegible] pleted of H antibody since no suitable strain was available which [illegible] wn to possess H antigen identical with CS 1483 However [illegible] ing the cultures on broth agar supplemented with 0 1 per ce[illegible] the formation of flagella was inhibited Prel grown cultures [illegible] lacking the K88 antigen (18 C cultures of K88 37 C cultur[illegible] K88 did not agglutinate either in the H10 serum or in the absor[illegible] um of CS1483 and flagella were

O149 K91 K88ac H10 strains are as follows rapid fermentation of adonitol sorbitol arabinose xylose rhamnose maltose sorbose mannitol (with gas) and glucose (with gas) delayed fermentation of salicin and non fermentation of dulcitol inositol and sucrose No liquefaction of gelatine no production of H S no growth in ammonium citrate the Voges Proskauer reaction is negative and the methyl red reaction is positive and nitrates are reduced Unlike other strains of *E coli* they hydrolyse urea The existence of urea positive O141 strains was mentioned previously (11 12) and also the fact (12) that CS1522 (an O149 strain see the introduction) decomposes urea Other authors have made similar observations regarding strains isolated from pigs indicating that the ability to produce urease might frequently be a special trait of *E coli* strains from pigs (cited by *Sojka* 1965)

DISCUSSION

In the present report evidence has been presented of the occurrence of a new B antigen numbered K91 in combination with K88ac antigen in a strain of *E coli* isolated from a pig Several porcine strains possessing two K antigens have been described previously but in this instance the fact was clearly demonstrated only after suppression of the H antigen which interfered with the determination of other antigens *Doll* (1) showed in 1956 that the anionactive detergent named Pril® (Bohm Fettchemie Dusseldorf) could effectively inhibit the swarming of *Proteus* bacteria without influencing the colony growth In studies with the electron microscope only fragments of flagella were visible on the bacteria after growth in pril supplemented media in contrast to the appearance in the control test

Cultures of CS1483 grown on broth agar medium prepared in the usual manner agglutinated to a high titre in a heterologous H specific serum agglutination was not obtained however in parallel tests with cultures grown on broth agar containing pril The appearances of preparations stained according to Leifson suggested that the effect of pril which has alkaryl sulphonate as its main constituent depends on the suppression of flagellar development As the addition of pril did not alter the O or B agglutination characteristics in homologous sera the usage of this material in the present investigations was considered to have no special disadvantages

The spectrum of activity of pril as an inhibitor of the H antigens of *E coli* was not investigated In gel diffusion tests on antigen antibody systems connected with quite a distinct *E coli* culture the disappearance of the H line and the development of an unidentified line have been observed simultaneously in association with the use of pril in culture media (unpublished results) It should be stressed therefore that the effect of pril on the serological behaviour of *E coli* cannot be fully assessed without further study

The present investigation provides an opportunity to consider certain anomalies relating to the system of designating the antigens of *E. coli*. The L antigen in CS1483 was recorded as K88ac, a known antigen sharing the common factor a with K88ab, another known antigen of the L variety. The B antigen was designated as K91, a newly assigned K number, since it was found to be unrelated to the known K antigens. The H antigen was recorded as H10; we know that some of the hitherto established H antigens are related, but when the H antigen of a test strain is listed as one of the known ones, it has not been common practice up to now to determine whether the two antigens in question are identically related. Thus we do not know how complex the H antigens really are. The O antigen in CS1483 was found to be different from all the established O antigens, although cross relationships were demonstrated with the O factors O1, O112ac, O117 and O144. We know that many established O antigens with different numbers are in fact related, but as the relationships have only in a few isolated instances been expressed as a, b, c, etc. antigen factors, we considered it worthless in the present case to express the O antigen of CS1483 as e. g. O144ac. The O112 group is one of the few O antigen groups already subdivided into two separate components, O112ab and O112ac. The strain CS1483, though distinct from O112ab, cross reacts with the O112ac component and could thus also be recorded as O112cd. The adoption of this system in expressing the O antigens of *E. coli* would, however, require extensive changes in the existing structure of the O antigen scheme. As no agreement has so far been reached as to the advantages or disadvantages of such a plan, it was considered more appropriate to give the O antigen of CS1483 a new number, viz. O149.

It has been stated repeatedly that the K88(L) antigen is not developed by cultivation at 18° C, while the different B antigens found in K88 strains have mainly been detected because they are not suppressed in cultures grown at this temperature. However, it should be stressed that the dependence on temperature cannot be considered as a general rule relating to development of B and L antigens.

SUMMARY

A new O antigen numbered O149 and a new K antigen of the B type numbered K91(B) have been established in *E. coli*. The antigens were found in combination with K88ac(L) antigen in strains isolated from diseased piglets both in D.D.R. and [illegible] and.

The supplementation of the [illegible] medium with 0.1 per cent [illegible] was found useful in [illegible] unusually strong development of flagella which impeded the [illegible] analysis of other surface antigens.

O149 K91 K88ac H10 strains are as follows: rapid fermentation of adonitol, sorbitol, arabinose, xylose, rhamnose, maltose, sorbose, mannitol (with gas) and glucose (with gas); delayed fermentation of salicin and non-fermentation of dulcitol, inositol and sucrose. No liquefaction of gelatine, no production of H_2S, no growth in ammonium citrate; the Voges-Proskauer reaction is negative and the methyl red reaction is positive and nitrates are reduced. Unlike other strains of *E. coli* they hydrolyse urea. The existence of urea-positive O141 strains was mentioned previously (11, 12) and also the fact (12) that CS1522 (an O149 strain, see the introduction) decomposes urea. Other authors have made similar observations regarding strains isolated from pigs, indicating that the ability to produce urease might frequently be a special trait of *E. coli* strains from pigs (cited by *Sojka* 1965).

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Cultures of CS1483 grown on broth agar medium prepared in the usual manner agglutinated to a high titre in a heterologous H specific serum. Agglutination was not obtained, however, in parallel tests with cultures grown on broth agar containing pril. The appearances of preparations stained according to Leifson suggested that the effect of pril, which has alkaryl sulphonate as its main constituent, depends on the suppression of flagellar development. As the addition of pril did not alter the O or B agglutination characteristics in homologous sera, the usage of this material in the present investigations was considered to have no special disadvantages.

The spectrum of activity of pril as an inhibitor of the H antigens of *E. coli* was not investigated. In gel diffusion tests on antigen-antibody systems connected with quite a distinct *E. coli* culture the disappearance of the H line and the development of an unidentified line have been observed simultaneously in association with the use of pril in culture media (unpublished results). It should be stressed, therefore, that the effect of pril on the serological behaviour of *E. coli* cannot be fully assessed without further study.

ADDENDUM

The serotype of the Irish Strain A1 (Abbotstown) was mentioned recently in a paper by *Sweeney* (8)

Strains isolated from swine of the serotype 0149 K*(B) K88ac(L) H10 were described in a Polish paper by *Kas ubkiewic et al* (3) in 1967 These strains had been examined serologically in the W H O International Escherichia Centre where the O antigen had been given the provisional number 0149 which has now become the established number At the time of that examination the B antigen had not yet received its own number

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TABLE 1

Delay of CPE after Addition of Different Dilutions of Non Heated GP Sera to Heated Sera (IS) from Rabbits Immunized with Adenovirus Type 7

	Delay of CPE in days					
	Non heated IS no GP serum added	Heated IS no GP serum added	Heated IS GP serum added			
			final dilution of GP serum			
			1 4	1 8	1 16	1 32
2 day IS (final dilution 1 2) + GP serum No 14	9	5	9	9	7	7
GP serum No 14 control			2	0	ND	ND
8 day IS (final dilution 1 128) + GP serum No 21	ND	2a	9	9	4	4
GP serum No 21 control			4	3	2	ND

ND = not done

a non heated sample was not tested simultaneously in an earlier experiment no difference between non heated and heated samples of the same serum diluted 1 128 was recorded

In all GP serum pools employed a slight background neutralization (*Svehag & Mandel* 1964) to adenovirus type 7 was demonstrated Non heated GP serum was used at dilutions which in themselves did not delay the cytopathic effect (CPE) more than a maximum of 3 days This usually meant a final dilution (before mixing with virus dilution) of 1 4 or 1 8 Further dilution resulted in a decreased restoring ability (Table 1) Heat inactivation of the GP sera often decreased the background neutralization

In rabbit sera collected three days or more after immunization a serum dilution of 1 2 was too potent to admit the registration of differences in neutralizing effect within the experimental period which was limited by the life span of the HeLa cell cultures Therefore sera collected on days 1–16 were tested in fourfold increasing dilutions starting from 1 4 (Fig 1 b–f) The effect of GP serum on the neutralizing activity of heated sera was very marked especially in immune serum dilutions of 1 64 and above In all sera collected on days 3–9 after immunization the addition of non heated GP serum caused a prolongation of 4 9 days in the delay of CPE

In the 9 day serum (Fig 1d f) there was a considerable difference between samples tested in the presence and in the absence of GP serum in dilutions as high as 1 1024 The same serum presents an example

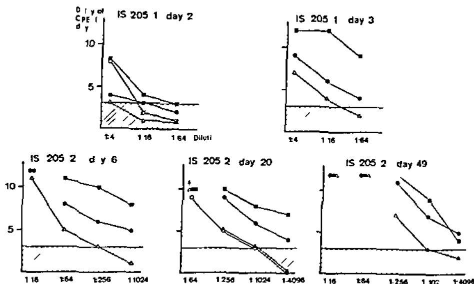


Fig 4

The development of neutralizing activity in rabbits immunized by several antigen injections (see text) For explanation of symbols see Fig 1

antigen in adjuvant followed by intravenous injections were also tested The first intravenous injection was given on day 10 After another three injections (on days 21 28 and 42) the animal was exsanguinated on day 49 A sequence of changes of neutralizing activity similar to that in animals immunized by a single injection was recorded (Fig 4) With *increasing intervals between the initial immunizing injection and the* collection of specimen the influence of heated GP serum gradually approached that of non heated GP serum

Time of Appearance of Mercaptoethanol Resistant Homotypic Antibody Activity

In the experiment with consecutive bleedings from the same animal (Fig 2) the sera collected up to day 4 had a mercaptoethanol sensitive neutralizing activity whereas sera from day 8 and onwards showed stability to mercaptoethanol treatment In other immunized rabbits tested all the 3 day sera and some 4 day sera were sensitive according to the standards given under Materials and methods However there were some few animals which had mercaptoethanol stable antibody activity already on the fourth day After the end of the first week all sera contained mainly antibodies totally resistant to mercaptoethanol treatment

Influence of GP Serum on Heterotypic Neutralization

Some sera from rabbits immunized with adenovirus type 7 antigen were tested for neutralization of adenovirus types 2 and 3 in the pre

sence and in the absence of GP serum Virtually no neutralizing capacity was demonstrated to type 2 The addition of GP serum caused a delay of one day in some instances A variation of only one day sometimes occurs also between duplicate cultures inoculated with identical mixtures On the other hand the usual cross reactivity of type 7 sera with type 3 antigen was recognized also in these experiments For comparison the delay of type 3 CPE was entered in Fig 1 d for the 9 day serum diluted 1 64 Maximal differences of up to 5 days were caused by addition of non heated GP serum to type 7 sera collected at different intervals after immunization The effect of heated GP sera developed in the same way in the heterotypic (type 3) as in the homotypic neutralization *i e* a gradual approach to the level of non heated GP serum effect was noted in later sera

Haemolytic Activity of GP and Rabbit Sera

For two of the three GP serum pools employed CH_{50} values were determined Serum pool No 16 tested after 16 months of storage contained approximately 192 CH_{50} units per ml and serum pool No 21 after 3 months of storage approximately 218 CH_{50} units per ml As the GP sera were added in a final dilution of 1 4 or most frequently 1 8 this means that immune sera were tested with 24–54 CH_{50} units per ml present Since equal parts of serum dilution and virus dilution were used for the neutralization mixture this contained about 12–27 CH_{50} units per ml Rabbit sera however were found to contain no more than approximately 10 CH_{50} units per ml of undiluted serum

DISCUSSION

The technique employed in the earlier investigations of the thermolability of adenovirus neutralizing activity (*Svartz Malmberg* 1963 1966) was originally developed for studies of adenovirus antibodies in patient sera which were tested only at a dilution of 1 2 (*Kjellen et al* 1957) When comparative studies of the influence of addition of guinea pig (GP) serum were carried out it was found that the potentiation of most early rabbit immune sera tested at low dilutions was less than expected This appears to be in accord with the findings by *Westaway* (1965) who showed that non heated serum might even inhibit neutralization by high concentrations of antibodies

On the other hand by adding non heated GP serum to early immune sera diluted 1 16–1 64 an enhancement of neutralizing activity was observed which was qualitatively comparable to the results obtained by *Yoshino & Taniguchi* (1965) However with increasing intervals between immunization and collection of serum a gradually increasing influence was obtained also with heated GP serum This is obviously not in agreement with the hypothesis that the complement system as a whole should be the potentiating factor complex Nor do the results

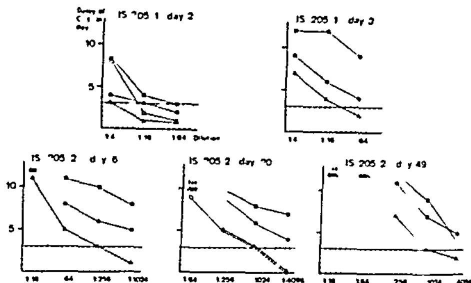


Fig. 4.
The development of neutralizing activity in rabbit immunized by several antigen injections (see text). For explanation of symbols see Fig. 1.

antigen in adjuvant followed by intravenous injections were also tested. The first intravenous injection was given on day 10. After another three injections (on days 21, 28 and 42) the animal was exsanguinated on day 49. A sequence of changes of neutralizing activity similar to that in animals immunized by a single injection was recorded (Fig. 4). With increasing intervals between the initial immunizing injection and the collection of specimen the influence of heated GP serum gradually approached that of non heated GP serum.

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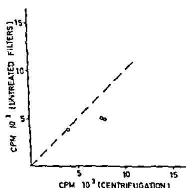


Fig 1

Comparison of filter harve t without hyamine treatment and harvest by centrifugatior Two aliquots of 1000 μl were taken from each of ten different lymphocyte cultures labelled with ³H thymidine

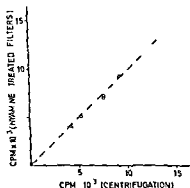


Fig 3

Comparison of filter harvest with hyamine treatment and harvest b centrifugation Two aliquots of 1000 μl were taken from each of fourteen different lymphocyte cultures labelled with ³H thymidine

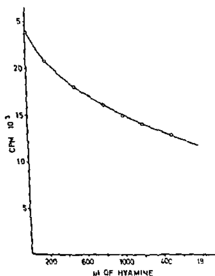


Fig ?

Quenching in luced by hy m ((µCi ³H thymidine counted in t l (ne sc ntillator with increa ing n ι t of hyamine added

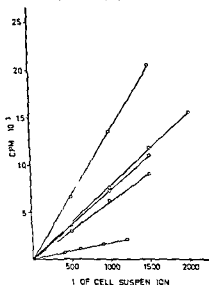


Fig 4

lati nshi between count rates and nber of labelled cells using f lter ⊦ est with hyamine treatment Fr n r of 6 different lymphocyte ul arying volumes were harves ed

Results

Without hyamine treatment In tc a f t rs th t had n t been treated with hyamine dioxane scintillat r gas , r t ng efficiency than tcluene scin till tor The acti it remaine l locali d n tl lters e en after se eral days in the scintillator solution The orientation f tn f ι r in the counting vial proved to be

in the 0.2[illegible] μ filtrate was significantly reduced by centrifugation at 66,000 G for 120 min. The reduction was 2–3 dilution steps in most of the experiments.

The present results constitute evidence that broth cultures of *M. pneumoniae* may contain considerable amounts of non-cell-bound complement fixing antigen. This antigen passes through filters which effectively retain viable cells and it is noncentrifugable at 66,000 G. The non-cell-bound antigen may be the result of degradation of dead plasma cells or it may be produced by living cells during metabolism and growth. Relevant to the latter hypothesis is *Bredt's* (1) observation that colonies of *M. pneumoniae* strain FH grown on glass surface produce "grape-like" structures growing into the broth. Further investigations on the occurrence and properties of the non-cell-bound complement fixing antigen of *M. pneumoniae* have been initiated.

TABLE 1

Filtration of Broth Culture of M. pneumoniae (FH) Strain through Millipore® Filters. Effects upon the Number of Viable Cells and upon the Complement Fixing Titre

	No. viable cells measured by					
	Change in colour in tube with dilution				No. colony forming units/ml	CF titre
	10^{-1}	10^{-2}	10^{-3}	10^{-4}		
Unfiltered broth culture	+	+	+		2.4 × 10^5	1:64
MF 0.65 μ filtrate	+	+			[illegible] 10^4	1:64
MF 0.[illegible] μ filtrate					0 (<20)	1:64
MF 0.[illegible] μ centrif.						1:8
MF 0.1 μ filtrate					0 (< [illegible])	1:64

The MF 0.22 μ filtrate was also tested in CFT after centrifugation at 66,000 G for 120 min.

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Acta path microbiol scand 75 513–519 1969

The Department of Anatomy and Histology and the Department of Paediatrics
Karolinska Institutet Stockholm Sweden

STUDIES ON CLOSURE OF THE DUCTUS ARTERIOSUS

V *Microradiographical Observations with Ultrasoft X rays on Closure of the Ductus Arteriosus in the Rat and the Guinea Pig*

By

P Yngve Hörnblad and Ulf Friberg

Received 26 viii 68

In our laboratory a whole body freezing technique has been used for morphological studies of the process of ductal closure (*Hornblad & Larsson* 1967a) In earlier investigations in this series the prenatal development of the ductus was studied and the ductal closure rate determined in a number of species (*Hornblad & Larsson* 1967b *Hornblad* 1967 1969)

It is now generally accepted that closure of the ductus arteriosus shortly after birth is brought about by a contraction of its muscle coat (*Moss et al* 1964) Yet there exists no plausible explanation of the eliciting mechanism In several investigations it has been demonstrated that a rise in blood or tissue oxygen tension will cause the ductus to contract (*Born et al* 1956 *Kovalčik* 1963 *Assali et al* 1963 *Reis & Anderson* 1964) This behaviour of the ductal muscle cells differs from that of smooth muscle cells in the pulmonary artery and aorta which do not react under the same conditions (*Kovalčik* 1963)

During closure of the ductus the thickness of the wall increases in relation to the decrease in internal diameter (*Hornblad & Larsson* 1967b) The gain in wall thickness is accompanied by a condensation and increased waviness of the elastic membranes and thickening of the interposed muscle cells The intimal layer is compressed and forms a solid core in the vessel In the guinea pig tissue defects occur particularly in the inner media (*Hornblad* 1967) In the present work these light microscopical observations were complemented by a qualitative microradiographical study of the structure of the ductal wall during closure This study comprised two species with different closure rates viz the rat and guinea pig In the rat a 50 per cent reduction in internal diameter occurs after about 30 minutes the ductus is fully

The authors are greatly indebted to Dr *K Sune Larsson* Department of Anatomy and Histology Karolinska Institutet for fruitful discussions The work was supported by funds from the *Swedish Medical Research Council* (14X–993–04 and 14Y–993 04) the *Association for the Aid of Crippled Children* New York the *King Gustaf V 80th Birthday Fund* and *Knut and Alice Wallenberg Foundation*

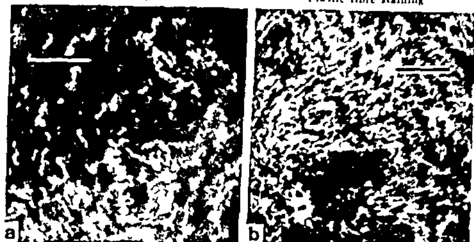


Fig 2

Cl ed ductus in the guinea pig Microradiogram exposed at 14 kV 50 mA 30 min Section *b* counterstained with haematoxylin and eosin × 185 —The internal elastic membrane shows a high dry mass. In other parts of the media the lamellar pattern is less distinct than in the rat Densely black areas represent tissue defects (arrow) (Closure 4 hrs)

could be observed in the media particularly along the inner border These corresponded to the distribution of elastic fibres in the resorcin fuchsin stained sections

During closure the contrast of the microradiograms increased with the emergence of a distinct lamellar pattern (Fig 1c and e) In the closed ductus radio opaque and radiolucent bands alternated The dense wavy bands corresponded to the condensed elastic lamellas The highest dry mass concentration occurred in the internal elastic membrane Intimal and muscular cells presented the same low density

In most of the microradiograms and stained sections the aorta could also be studied The elastic lamellas of the aorta had a greater dry mass and showed heavier staining than those of the ductus No difference between the aorta and the ductus could be observed with respect to density of the muscle cells

Guinea pig The microradiographical appearance of the ductus was similar to that in the rat However the lamellar pattern of the closed ductus was less distinct with the exception of the internal elastic membrane (Fig 2) Stained sections also showed the elastic lamellas to be less prominent than in the rat The characteristic tissue defects were readily identifiable as areas with minimal density in the microradiograms

In the aorta the elastic lamellas were more prominent both in microradiograms and stained sections than in the ductus This difference was more pronounced than in the rat

DISCUSSION

The present paper is based on the use of the high resolution technique for contact microradiography with ultrasoft X rays developed mainly by Engstrom and co workers (*Greulich & Engstrom* 1956 *Engstrom et al* 1957) With this technique it is possible to achieve a resolution approaching that of the light microscope The contrast in the microradiographical image is determined by the differential absorption in the tissue structures examined In soft tissues X ray absorption is caused mainly by carbon nitrogen and oxygen Hence X ray absorption may be used as a quantitative measure of the organic dry mass of tissue structures The microradiographical technique is also useful in qualitative experimental morphology as an adjunct to light microscopy (*e g Greulich* 1960 *Travis & Friberg* 1963a b *Oberg* 1964) It is from the latter aspect that this technique was applied in the present study on ductal closure

In both species examined the most conspicuous event during closure was the condensation of the elastic lamellas This was very strikingly visualized in the microradiograms The high dry mass of elastic membranes is in agreement with the observations by *Greulich* (1960) and can be interpreted as indicating a high concentration of protein On the other hand the dry mass was uniformly low in the interlamellar media and in the intima and did not change during the period of study

The condensation of the elastic lamellas and the absence of changes in dry mass of other structures in the wall corroborate the prevalent concept of ductal closure as being caused by muscular contraction These findings do not on the contrary support certain supplementary theories of ductal closure For instance it has been suggested that angiomalacia with dissociation of the inner media precedes closure and is a prerequisite for effective occlusion (*Meyer & Simon* 1960 *Hoffman* 1964) It has also been proposed that deposition of mucopolysaccharides in the wall is a factor in closure (*Sciacca & Condorelli* 1960 *van Ingen* 1964) In either case it seems reasonable to assume that the related tissue changes would characteristically affect dry mass distribution There was however no indication in the microradiograms of any such changes

Recent observations in several species have shown that ductal closure is in general terms accompanied by the same structural changes in the wall regardless of differences in closure rates (*Hörnblad* 1967) No[illegible] have any significant differences in the structural changes been [illegible] served by microradiography between two species representing [illegible] and fast closure rates respectively All these [illegible]mentary mor[illegible] logical studies clearly indicate the importan[illegible] of [illegible] muscular co[illegible] tion for ductal closure It therefore seems [illegible] to devote [illegible] investigatory efforts to experimental interf[illegible] with normal

Acta path. microbiol. scand. 75: 570–576, 1969

The University Institute for Pathological Anatomy and The Department of Surgical Research, Rikshospitalet, Oslo, Norway

IN VITRO UPTAKE OF OESTRADIOL IN DMBA INDUCED BREAST TUMOURS OF THE RAT

By

STEN SANDER

Received 23.ix.68

Previous studies have shown that breast tumours induced in female rats by administration of 7,12-dimethylbenz(a)anthracene (DMBA) have a similar affinity for oestradiol as the uterus and the vagina (4, 5). A further similarity is that the oestradiol is retained essentially unconverted in these tissues (1, 7).

In the majority of the DMBA tumours growth is dependent on the presence of ovarian hormones, while in some growth is not affected by ovariectomy. Theoretically, this difference may be due either to an increased incorporation of oestradiol in the responsive tumours, or to an increased sensitivity of these tumour cells to the hormone. Previous *in vivo* investigations (6, 8) indicate that there is in fact a correlation between the response of the tumours to ovariectomy and their uptake of oestradiol. The hormone-responsive DMBA tumours concentrate oestradiol to a higher degree than the hormone-unresponsive tumours.

In the present study an attempt has been made to establish an *in vitro* method which differentiates hormone-responsive from hormone-resistant tumours. This is also of clinical interest regarding the choice of endocrine therapy in human breast cancer.

Jensen et al. (1) have found that slices of hormone-responsive DMBA tumours concentrate oestradiol during *in vitro* incubation. In a previous paper (7) it was reported that normal breast tissue also accumulates oestradiol during incubation. In the present study the uptake of oestradiol in DMBA tumours and in corresponding normal tissue is compared. A correlation is also made between the DMBA tumours' response to oophorectomy and their ability to accumulate oestradiol *in vitro*.

Furthermore, uptake of oestradiol in DMBA tumours can be markedly inhibited by an oestrogen antagonist, Upjohn U 11 100 (1, 4). This effect has been introduced as a criterion for the specific oestradiol uptake and binding in target organs (3, 4). Therefore the DMBA tumours' ability to take up oestradiol in the presence and absence of

This work has been supported by *The Norwegian Society for Fighting Cancer* (Norsk Forening til Kreftens Bekjempelse, Oslo).

Dr. *Sverre Skrede* is gratefully acknowledged for stimulating discussions.

U 11 100 was also studied The effect of the inhibitor on normal breast tissue was investigated under similar conditions

The mechanism involved in the incorporation of oestradiol might be elucidated by studying the effect of metabolic inhibitors Therefore the effect of some inhibitors on the oestradiol uptake in DMBA tumours and in normal breast tissue was also investigated

Finally the viability of tumour tissue slices was studied by measuring their consumption of oxygen during the incubation period

MATERIALS AND METHODS

Breast tumours were induced in female Wistar rats by intravenous injection of 3 mg of 7 12 dimethylbenz(a) anthracene when the rats were 50 days old The injection was repeated after 3 days Only growing tumours were examined When the tumours were 1 5–2 cm in diameter oophorectomy was carried out through a small midline incision under ether anaesthesia The tumours were measured through the skin with calipers 6–10 days after the operation the tumours were classified as hormone responsive or hormone unresponsive as previously described (8) Normal breast tissue was taken from pregnant rats (18–20 days pregnant) which had been subjected to ovariectomy and hysterectomy 5 days before the experiment The animals were killed by decapitation and breast tumour and breast tissue were rapidly taken out Pieces of the pectoralis major muscle were removed and used as a reference in every experiment Great care was taken to excise possible necrotic areas from the tumours The tissues were sliced on a Stadie Riggs microtome and approximately 40–50 mg of tissue were put into each incubation flask. Three parallels were run from each tumour Each flask contained 4 ml of Krebs Ringer phosphate buffer pH 7 4 The buffer was prepared immediately before use from stock solutions and tritium labelled oestradiol 17β 4 $\times$ 10^{-5} μg per ml medium) was added to each flask The incubation was carried out with continuos shaking at 37 C for 2 hours

For inhibition of oestradiol uptake Upjohn 11 100 was used in a concentration of 10^{-5} M in the medium The other inhibitors studied were used in the following concentrations in the medium Iodoacetate 10^{-3} M G strophantin 2 $\times$ 10^{-5} M Potassium cyanide 10^{-3} M 2 4 dinitrophenol 10^{-4} M and oligomycin 1 μg/ml medium

Measurement of radioactivity and determination of protein in the tissue slices were performed as previously described (8) The uptake of radio ctivity in the tissue is expressed as D P M per mg of protein The ratio between concentration of radioactivity in tumour tissue and muscle control was calculated for each tumour The radioactivity in the medium was determined after ether extraction Tissue for histological examination was embedded in paraffin and sections stained with haematoxylin/eosin

Determination of oxygen consumption by the tumour slices was performed as previously described (7)

The statistical evaluation is based on Students t test (Mr H Kvaase)

RESULTS

The correlation between oestradiol uptake and castration response in DMBA tumours is shown in Table 1 The tumour muscle ratio was found to be in the range 1 1 1 7 in all the hormone unresponsive tumours examined Thus these tumours did not concentrate the labelled oestradiol to any significant extent On the other hand the majority of hormone responsive tumours showed a high tumour muscle ratio indicating a more pronounced oestradiol affinity The ratio of some tumours in this group were however in the observed range of ratios for hormone unresponsive tumours

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The University Institute for Pathological Anatomy and The Department of Surgical Research Rikshospitalet Oslo Norway

IN VITRO UPTAKE OF OESTRADIOL IN DMBA INDUCED BREAST TUMOURS OF THE RAT

By

STEN SANDER

Received 23 ix 68

Previous studies have shown that breast tumours induced in female rats by administration of 7 12 dimethylbenz(a)anthracene (DMBA) have a similar affinity for oestradiol as the uterus and the vagina (4 5) A further similarity is that the oestradiol is retained essentially unconverted in these tissues (1 3)

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mone unresponsive cancer tissue and in the corresponding normal tissue. It was found that normal breast tissue as well as DMBA induced breast tumours possessed some ability to take up oestradiol *in vitro*. There was, however, a distinct difference in the oestrogen affinity. After incubation with tritium labelled oestradiol the average tumour/muscle ratio was significantly higher in the hormone responsive tumours than in the hormone unresponsive ones. There was, however, some overlapping between the two groups since three out of 14 hormone responsive tumours had values in the range observed in hormone unresponsive tumours.

The findings suggest that a high uptake of oestradiol in DMBA tumours prognosticates regression after castration. On the other hand tumours with a low oestradiol uptake are expected to continue their growth after castration. Some tumours with a low oestradiol uptake may, however, respond favourably to castration.

Jungblut et al. (4) have shown that the uptake of oestradiol can be reduced by U 11 100 in hormone responsive tumours. This effect is only found in target tissues for oestradiol. The results presented in this study confirm that the oestradiol uptake can be reduced by U 11 100 in a hormone-responsive DMBA tumour, whereas the inhibitor was essentially without effect in a hormone unresponsive tumour. U 11 100 also lowered the uptake of oestradiol markedly in normal breast tissue as it does in other oestrogen responsive tissues of the rat. Thus hormone responsive DMBA tumours and normal breast tissue both appear to have a specific oestradiol uptake. If the uptake of oestradiol in target organs depends on the presence of protein receptors (10) one must assume that hormone responsive DMBA tumours as well as normal breast tissue possess active receptor groups. On the other hand binding sites for oestradiol are probably not accessible to the same extent in hormone unresponsive tumours.

The observed effects of the metabolic inhibitors suggest possible mechanisms for the incorporation of oestradiol in the examined tissues. Iodoacetate was found to reduce the oestradiol uptake significantly. Iodoacetate in the concentration used (10^{-3} M) will interfere with cellular membranes in general. The observed effect of iodoacetate is probably mediated by interference with cellular SH groups (11). It therefore appears likely that intact SH groups are required for an accumulation of oestradiol within the cells of normal breast tissue and breast tumours. The other metabolic inhibitors used in this study did not significantly influence the uptake of oestradiol.

Since G strophantin had no effect, oestradiol uptake in the tissue is probably not linked to the sodium potassium transport system. Furthermore energy made available by oxydative phosphorylation appears not to be required for the binding of the hormone since inhibitors of oxydative phosphorylation such as oligomycin, uncoupling agents (2,4-DNP) or respiratory inhibitors (KCN) did not lower the accumulation

of oestradiol The results set out in Table 3 suggest that there is no energy linked incorporation of oestradiol across the cell membrane

The effect of various sulphydryl blocking reagents on the uptake of oestradiol in the uterus has been studied by *Terenius* (9) and *Jensen et al* (2) They found that the ability of the uterus to take up oestradiol was lost after treatment of the tissue with N ethylmaleimide mercuri benzoates and iodoacetate The results obtained in the present study suggest that the mechanism involved in uptake and binding of oestradiol in breast tissue and DMBA induced breast tumours also depends on intact sulphydryl groups

Furthermore oxygen consumption by the tumour slices was determined as an index of viability There was a continous consumption of oxygen for at least three hours The oxygen uptake in tumour tissue was substantially higher than that previously observed in normal breast tissue (7) This may be explained by the higher cellularity of tumour tissue but it may also partly be due to a higher metabolic activity in tumour tissue The reduced consumption of oxygen observed when glucose was added to the medium (Crabtree effect) was of approximately the same degree in tumour and normal breast tissue

The results of the present investigation suggest a simple and practical way of determining the presence of active binding sites for oestradiol in breast tumours It is felt that the uptake of hormones by the tumour tissue may give clues as to the individual response of breast cancers to endocrine treatment *In vitro* measurements of oestradiol uptake in tumour slices as described in this study may therefore become of practical use for future routine examinations

SUMMARY

The uptake of oestradiol (6 7 ^{3}H) 17β in hormone responsive and hormone unresponsive breast tumours was studied after incubation in Krebs Ringer phospate buffer for 2 hours As previously found *in vivo*, the hormone responsive tumours tend to accumulate oestradiol to a relatively high degree Normal breast tissue also possesses some ability to accumulate oestradiol *in vitro* The high uptake of oestradiol in hormone responsive tumours can be significantly inhibited by the oestrogen antagonist Upjohn U 11 100 and similarly U 11 100 lowered the uptake in normal breast tissue This effect was not apparent in hormone unresponsive tumours

The effect of some metabolic inhibitors on the *in vitro* uptake of oestradiol was studied Iodoacetate in a concentration of 10^{-3} M lowered the uptake of oestradiol significantly both in normal breast tissue and in breast tumour The other inhibitors used in this study were essentially without effect The possible importance of intact SH groups for the incorporation of oestradiol in these tissues is discussed

A continuous consumption of oxygen was found during the incubation

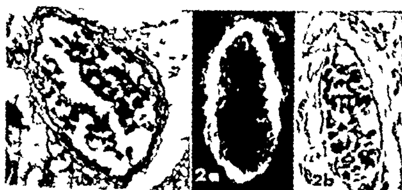
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Figs 1–2

Fig 1 Normal arteriole The internal elastic membrane is continuous around the whole circumference *Orcein* staining Magnification *400* ×

Fig 2 a Dilatation with deposition of fluorescent proteins *in* the wall b The same dilatation as a in orcein staining The internal elastic membrane *is* severely destroyed Magnification 400 ×



Fig 3

a Dilatation without deposition of plasma components in the wall The internal elastic membrane is continuous around the whole circumference *b* Another dilatation without deposition of plasma components in the wall demonstrating small discontinuations of the internal elastic membrane Orcein staining Magnification *400* ×

fluorescent proteins had penetrated into the arteriolar wall the internal elastic membrane in regular cross sections of the dilatation was either continuous along the circumference of the dilatation or had small discontinuations but never the heavily disconnected appearance as in dilatations in which fluorescent proteins or colloidal carbon particles had penetrated into the wall (Figs 3a and 3b)

The experimental groups were as follows

1 Two normal controls From these 2 rats 10 arteriolar segments were isolated for examination of the internal elastic membrane

2 Six normal rats pretreated with intravenous injection of 65 mg of Lissamine Rhodamine labelled porcine gamma globulins and followed by repeated intravenous injections of angiotensin (1 microgram per injection) in the course of 2 hours the intervals between the injections being about 5 minutes

3 Three normal rats receiving repeated intravenous injections of angiotensin (1 microgram per injection) in the course of 2 hours followed by intravenous injection of 0 1 ml of colloidal carbon particles From group 2 and 3 15 constrictions 22 dilatations permeable for either fluorescent proteins or colloidal carbon particles and 22 dilatations nonpermeable neither for fluorescent proteins nor colloidal carbon particles were isolated

4 A Five normal rats receiving only 1 intravenous injection of angiotensin (1 microgram) followed by intravenous injection of 0 1 ml of colloidal carbon particles when the blood pressure reached the maximal value 6 dilatations permeable for colloidal carbon particles and 8 nonpermeable dilatations were isolated

4 B: In 7 experiments an attempt was done to reduce the large dosis of angiotensin used in group 4 A but it was impossible to elevate the blood pressure sufficiently to bring about production of dilatations and constrictions along the course of the mesenteric arterioles with doses of angiotensin less than 750 ng per injection In the present experiments a rise of blood pressure from about 85 mm Hg to about 145 mm Hg was required for the manifestation of dilatations and constrictions The usual blood pressure increment following intravenous injection of angiotensin was 15 mm Hg after 100 ng 25 mm Hg after 200 ng and 45 mm Hg after 600 ng of angiotensin

5 Two normal rats receiving repeated intravenous injections of angiotensin (1 microgram per injection) in the course of 30 minutes followed by intravenous injection of both 35 mg of Lissamine Rhodamine labelled porcine-gamma globulins and 0 1 ml of colloidal carbon particles 10 dilatations with deposition of colloidal carbon particles in the wall were isolated

6 Three renal hypertensive rats studied 2 or 3 months after clamping of the left renal artery with a silver wire 0 18 mm internal width The rats were injected intravenously with 35 mg of Lissamine Rhodamine labelled porcine gamma globulins or 0 1 ml of colloidal carbon particles 20 minutes later a loop of the small intestine was fixed immediately 42 dilatations were isolated

RESULTS

The arterioles towards which the attention was directed were 60–100 micron in diameter measured *in vivo*

Group 1 (Controls) In all sections of 10 arteriolar segments the internal elastic membrane was recognized as a continuous jagged structure along the whole circumference of the arteriole (Fig 1)

Group 2 and 3 (Normal rats receiving repeated injections of angiotensin in the course of 2 hours) In all 22 dilatations permeable for either fluorescent proteins or colloidal carbon particles the internal elastic membrane was destroyed in such a way that it either was presented as disconnected fragments or was lacking in a big part of the circumference of the dilatation and disconnected in the remaining part of the circumference (Figs 2a and 2b) In 1 very long dilatation it was found that the internal elastic membrane was continuous around the whole circumference in that part in which no colloidal carbon particles had penetrated into the wall while it was destroyed in that part in which a penetration of colloidal carbon particles had taken place

In all 22 dilatations in which neither colloidal carbon particles nor

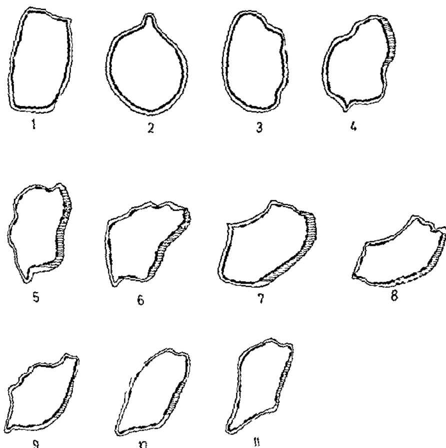


Fig [illegible]

Drawing of serial section ([illegible] micron thick) of a dilatation with deposition of both fluorescent protein and colloidal carbon particles in the wall. The deposition of fluorescent proteins is [illegible] and the deposition of colloidal carbon particles is dotted. It is seen that the deposition of fluorescent proteins is extending more proximal and distal and more circumferential than the deposition of colloidal carbon particles and that fluorescent proteins in [illegible] are deposited behind a part of the internal elastic membrane which is apparently continuous corresponding to the deposition of the fluorescent protein. In this figure such a part of a permeable dilatation has been chosen in which colloidal carbon particles only have deposited in a small part of the circumference of the wall while fluorescent proteins have deposited in a big part of the circumference. Magnification 175 ×

direction of the vessel. In some dilatations, however, the fluorescent proteins were found to be deposited not only corresponding to the deposition of the colloidal carbon particles but furthermore to areas exceeding those of carbon deposition in proximal-distal as well as in circumferential direction. Corresponding to the deposition of the colloidal carbon particles the internal elastic membrane was always destroyed, but corresponding to the deposition of the fluorescent [illegible]

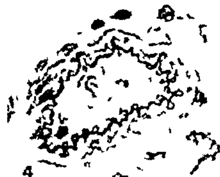


Fig 4
Constriction The internal elastic membrane is continuous Orcein staining Magnification 400 ×

In all 15 constrictions the internal elastic membrane was recognized as a continuous jagged structure along the whole circumference of the arteriole (Fig 4)

Group 4 A (Normal rats receiving only 1 intravenous injection of angiotensin) In the 6 dilatations in which colloidal carbon particles had penetrated into the wall the appearance of the internal elastic membrane was quite as described above in groups 2 and 3 concerning permeable dilatations

In the 8 dilatations nonpermeable for colloidal carbon particles the internal elastic membrane was recognized quite as described above in groups 2 and 3 concerning nonpermeable dilatations

Using colloidal carbon particles or fluorescent proteins as tracers for the penetration of the native plasma proteins into the wall of the dilatations it was found that the colloidal carbon particles very often penetrated as a broad belt deposited in ⅓ to ½ of the circumference of the dilatation while the deposition of the fluorescent proteins sometimes included 1/10 of the circumference corresponding to a very slender zone in proximal distal direction Therefore it was of interest to examine how colloidal carbon particles and fluorescent proteins deposited together in the wall of the dilatation when they were injected intravenously at the same time

Group 5 (Normal rats receiving repeated injections of angiotensin in the course of 30 minutes followed by intravenous injection of both fluorescent proteins and colloidal carbon particles) The 10 dilatations with deposition of colloidal carbon particles in the wall were examined first under the fluorescent microscope for examination of the deposition of the fluorescent proteins in the wall of the dilatation and subsequently under the light microscope after orcein staining for a comparison of the deposition of the colloidal carbon particles in the wall of the dilatation with the deposition of the fluorescent proteins In about half of the dilatations correspondance between the deposition of colloidal carbon particles and the deposition of fluorescent proteins was found to be very close both in proximal distal and in circumferential

only one intravenous injection of angiotensin In these permeable dilatations the internal elastic membrane was destroyed In this way it was determined that the destruction of the internal elastic membrane took place very early in the development of the hypertensive damage of the arterioles and that such a destruction was correlated to a penetration of plasma components into the arteriolar wall These results open the question how this correlation between penetration of plasma components into the wall of the dilatations and the destruction of the internal elastic membrane should be interpreted?

Three explanations are possible

1 The internal elastic membrane is destroyed by the high intraluminal blood pressure in the dilatations before penetration of plasma components into the intima takes place thus the destruction is a contributory cause why plasma components penetrate into the intima of the arteriole as concluded by *Lansing* 1959 from his studies on the development of arteriosclerosis in arteries This view is supported by an electron microscopic study on The early effect of hypertension on the aortic intima of the rat (*Still* 1967) In his study it was shown that four hours after constriction of the abdominal segment of aorta infiltration of mononuclear cells from the blood into the intima took place together with an accumulation of plasma elements in the subendothelial space The plasma elements appeared to gather in areas of intima which were contiguous to gaps in the internal elastic membrane

In the present experiments it was impossible to recognize under the light microscope or under the fluorescent microscope a penetration of the intravenously injected plasma components exclusively into the intima When a deposition of fluorescent proteins or colloidal carbon particles was recognized in the arteriolar wall this deposition was always situated both in the intima and in the media and partly in the adventitia In all these cases the internal elastic membrane was destroyed and in this way it is concluded that a penetration of plasma components from the intima to the media was related to "gaps in the internal elastic membrane exactly as described by *Ooneda et al* 1965 and *Kojimahara* 1967 The present results cannot answer the question whether a destruction of the internal elastic membrane might also influence the penetration of plasma components into the intima

2 The internal elastic membrane is destroyed either by the pressure exerted by the penetrating plasma components on the internal elastic membrane or by enzymes for instance elastas contained in the penetrating plasma components It is not probable that the internal elastic membrane is destroyed by the pressure exerted by the penetrating plasma components on the internal elastic membrane because the pressure gradient is presumably steep across the internal elastic membrane so that it is to be expected that the plasma components will disperse inside the intima luminal to the internal elastic membrane rather than destroying it A further argument against such a mechanism is that it

it was sometimes seen in one or two sections that the *fluorescent* proteins had deposited behind an undestroyed internal elastic membrane while the internal elastic membrane was *destroyed again in the* neighbour sections in which fluorescent proteins were also deposited (Fig. 5)

Group 6 (The renal hypertensive rats) From the 3 renal hypertensive rats (blood pressure 190 200 mm Hg) 42 dilatations were isolated None of these dilatations were permeable for the two intravenously injected plasma components but in the pancreas and the untouched kidney from one rat one arteriole in each of these organs was found permeable for fluorescent proteins In PAS stained sections it was recognized that the walls of these arterioles were necrotic with loss of the nuclei and destruction of the internal elastic membrane corresponding to the deposition of the fluorescent proteins Hypertensive damage was found in 9 of the 42 dilatations from mesenteric arterioles Five of these 9 dilatations had necrosis with loss of the nuclei in the wall or PAS positivity without necrosis and the remaining 4 dilatations had hyperplasia of the intima In the 5 dilatations in which PAS positivity was found both in the intima and the media the internal elastic membrane was destroyed In 1 of the 5 dilatations many PAS positive zones were found separated from each other by PAS negative zones Corresponding to the PAS positive locations the internal elastic membrane was destroyed while the internal elastic membrane was continuous corresponding to the PAS negative zones

In the 4 dilatations in which hyperplasia of the intima was found without PAS positivity the internal elastic membrane was continuous along the whole circumference

In 33 out of the 42 isolated mesenteric arteriolar dilatations no hypertensive damage was found in the wall Twenty of these 33 dilatations were cut in regular cross sections and in these sections the internal elastic membrane was found to be continuous or with small discontinuations along the circumference just as described above in groups 2 3 and 4A

DISCUSSION

It has been mentioned in the literature that the internal elastic membrane is more or less destroyed in arterioles with chronic hypertensive damage (*Race & Peschel* 1954 *Kojimahara* 1967) In their experiments it was not possible to determine in which phase of the development of the hypertensive damage of the arteriolar wall the destruction of the internal elastic membrane took place Nor was it possible to estimate the consequence of a destruction of the internal elastic *membrane for* the development of the hypertensive damage of *the arterioles* In the present work the arteriolar dilatations were seen to be permeable for intravenously injected colloidal carbon particles even in relation *to*

SUMMARY

From rats with acute hypertension dilated *mesenteric arterioles permeable* for plasma components were isolated for examination of the internal elastic membrane in orcein stained histological sections. In these permeable dilatations the internal elastic membrane was found to be destroyed in such a way that it presented itself either as disconnected fragments or was lacking in a big part and disconnected in the remaining part of the circumference of the dilatations.

In dilated mesenteric arterioles *nonpermeable* for plasma components the internal elastic membrane was found either completely continuous along the circumference of the dilatation or with only small discontinuations but never the heavily disconnected appearance as in dilatations in which plasma components had penetrated into the wall.

In mesenteric arterioles from *normal* rats the internal elastic membrane was found as a continuous jagged structure along the circumference of the arteriole. Similarly the internal elastic membrane was found as a continuous jagged structure along the circumference of the *constricted* arterioles from hypertensive rats.

The possible causal relationship between destruction of the internal elastic membrane and penetration of plasma components into the arteriolar wall is discussed.

Using colloidal carbon particles and fluorescent proteins injected intravenously at the same time as tracers for the native plasma proteins in the development of hypertensive damage of arterioles it was found *in some dilatations that the fluorescent proteins were deposited* partly corresponding to the deposition of the colloidal carbon particles and partly proximal distal and more circumferential to the deposition of the colloidal carbon particles. This finding indicates that the fluorescent proteins have either diffused inside the wall of the dilatation from an entrance common to the fluorescent proteins and the colloidal carbon particles or have been able to penetrate more easily than the colloidal carbon particles.

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is possible to find small discontinuations of the internal elastic membrane in dilatations which are apparently nonpermeable for plasma components. Nor is it probable that the internal elastic membrane is destroyed by an action of elastase on it because the enzyme in that case has to effect the destruction very quickly because the internal elastic membrane was also found to be destroyed in dilatations observed in the very short experiments described in group 4 A. Also in this case a further argument against such a mechanism is the fact that it is possible to find small discontinuations of the internal elastic membrane in dilatations apparently nonpermeable for plasma components.

3. The penetration of plasma components into the intima of the dilatations and the correlated destruction of the internal elastic membrane are independent effects of a common cause (the elevated blood pressure?).

At the present time it is impossible to establish the true explanation.

It was an unexpected finding that fluorescent proteins or colloidal carbon particles did not penetrate into the wall of the 5 dilatations from the renal hypertensive rats presenting PAS positivity and necrosis in the wall, whereas such a penetration into the pancreatic and the renal arteriole with necrosis in the wall took place. An explanation of this finding is very difficult but perhaps the previously penetrating native plasma proteins had "clotted" the penetration pathways in the mesenteric dilatations but not in the pancreatic and the renal arteriole.

Using colloidal carbon particles and fluorescent proteins injected intravenously at the same time as tracers for the native plasma proteins in the development of hypertensive damage of arterioles, it was found in some dilatations that the fluorescent proteins were deposited partly corresponding to the deposition of the colloidal carbon particles and partly proximal, distal and more circumferential for the deposition of the colloidal carbon particles. The finding that fluorescent proteins were found at places where colloidal carbon particles had deposited makes it probable that the mechanism by which the fluorescent proteins and the colloidal carbon particles penetrate into the wall of the dilatation is the same. The finding that the fluorescent proteins could be deposited more proximal and distal and more circumferential in the arteriolar wall than the colloidal carbon particles indicates that the fluorescent proteins have either diffused inside the wall of the dilatations from an entrance common to the fluorescent proteins and the colloidal carbon particles, or have been able to penetrate more easily than the colloidal carbon particles. In the 5 micron thick serial sections it can not be ruled out that the fluorescent proteins deposited behind an apparently undestroyed part of the internal elastic membrane may have penetrated from the intima to the media through "gaps" in the internal elastic membrane of such a small diameter that they were impossible to recognize in the light microscope.

SUMMARY

From rats with acute hypertension dilated mesenteric arterioles *permeable* for plasma components were isolated for examination of the internal elastic membrane in orcein stained histological sections. In these *permeable dilatations the internal elastic membrane* was found to be destroyed in such a way that it presented itself either as disconnected fragments or was lacking in a big part and disconnected in the remaining part of the circumference of the dilatations.

In dilated mesenteric arterioles *nonpermeable* for plasma components the internal elastic membrane was found either completely continuous along the circumference of the dilatation or with only small discontinuations but never the heavily disconnected appearance as in dilatations in which plasma components had penetrated into the wall.

In mesenteric arterioles from *normal* rats the internal elastic membrane was found as a continuous jagged structure along the circumference of the arteriole. Similarly the internal elastic membrane was found as a continuous jagged structure along the circumference of the *constricted* arterioles from hypertensive rats.

The possible causal relationship between destruction of the internal elastic membrane and penetration of plasma components into the arteriolar wall is discussed.

Using colloidal carbon particles and fluorescent proteins injected intravenously at the same time as tracers for the native plasma proteins in *the development of hypertensive damage of arterioles* it was found in some dilatations that the fluorescent proteins were deposited partly corresponding to the deposition of the colloidal carbon particles and partly proximal, distal and more circumferential to the deposition of the colloidal carbon particles. This finding indicates that the fluorescent proteins have either diffused inside the wall of the dilatation from an entrance common to the fluorescent proteins and the colloidal carbon particles or have been able to penetrate more easily than the colloidal carbon particles.

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Five treated animals were used. They were killed 5 months after thyroidectomy together with 2 untreated controls. About one half of the pituitary gland was used for light microscopy and the other half for electron microscopy. The thyroid regions of the ^{131}I treated mice were also saved for light microscopy to check the effect.

For light microscopy the pituitary tissue was fixed in 4 per cent neutral buffered formaldehyde solution and stained with trichrome periodic acid Schiff according to *Pearse* (1960).

For electron microscopy the tissue was fixed in glutaraldehyde (*Sabatini et al.* 1963) followed by post fixation in osmium tetroxide solution. The tissue was embedded in Epon (*Luft* 1961) and sectioned with glass knives on an LKB microtome. The sections were double stained first using uranyl acetate and then lead citrate (*Reynolds* 1963). They were examined in a Siemens Elmiskop I A or a Zeiss EM 9 microscope both operated at 60 kV with a 50 μ objective aperture.

RESULTS

At autopsy all the treated animals showed an enlarged pituitary gland. No tumour was seen. The dose of ^{131}I used had completely destroyed the thyroid gland.

Light Microscopy

The histological appearance of the pituitary gland in the treated animals is shown in Fig. 1. There was a diffuse hyperplasia of the anterior pituitary in all treated animals. There were no signs of tumour or adenomatous hyperplasia. The cell picture was dominated by enlarged cells of two types. One cell type had large nuclei often with prominent

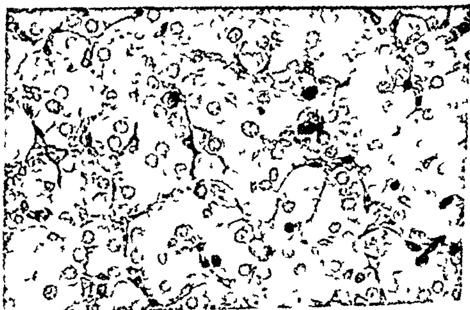


Fig. 1.

Light microscopic picture of the pituitary gland. Two predominating cell types are seen. The large cells with a pale vacuolated cytoplasm are thyroidectomy cells. The smaller rounded cells with grey cytoplasm are *acidophils* with a varying amount of stainable acidophil granules in the cytoplasm. Trichrome PAS × 600.

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nucleoli and an abundant foamy and frequently vacuolized cytoplasm The cytoplasm was essentially unstained and mostly without visible secretory granules Occasional cells showed a few coarse PAS positive granules in the cytoplasm The cells of the other type were somewhat smaller with pale nuclei often with a prominent nucleolus The cytoplasm contained varying amounts of stainable acidophil granules Most of the acidophil cells were very sparsely granulated

A few anterior pituitary cells of ordinary appearance—small chromophobes densely granulated acidophils PAS positive basophils—were scattered among the enlarged cells

Electron Microscopy

The cytology of the anterior pituitary gland was essentially similar in all treated animals Two different cell types were predominant among the anterior pituitary cells (Fig 2) One cell type (Figs 2 and 3) was characterized by large cells with uniformly granulated nuclei containing one or more prominent nucleoli The endoplasmic reticulum was abundant in the form of small rounded or larger irregularly shaped cisternae The cisternae contained a finely granulated material of low density Ribosomes were numerous either free in the cytoplasm or attached to the membranes of the endoplasmic reticulum The mitochondria were generally numerous and many of them large and elongated Branched mitochondria were frequently found The conspicuous Golgi complex was made up of flattened sacs vacuoles and vesicles The secretory granules were few often concentrated to the Golgi region or near the cell margins They measured up to about 90 mμ in diameter In many cells the cisternae of the endoplasmic reticulum contained dense granules resembling secretory granules Cytoplasmic bodies were observed in the cytoplasm in the form of multivesicular bodies occasional lipid droplets and membrane bound dense bodies

The cells of the other predominating type (Figs 2 and 4) were somewhat smaller They had a nucleus with one or more prominent nucleoli The cytoplasm showed a highly developed rough s[illegible]d endoplasmic reticulum in the form of parallel arrays In many of the cells the endoplasmic reticulum showed whorl like figures [illegible] formations composed of concentric [illegible]ired membranes of [illegible] surfaced endoplasmic reticulum Th[illegible] complex was [illegible] extensive The cytoplasm contained secretory granules in varying amounts Most of the cells were sparsely granulated The secretory granules had a characteristic appearance being [illegible] or irregularly shaped They measured up to about 200 mμ in their shorter dimension and up to about 400 mμ in their longer dimension The cytoplasm of the cells often contained cytoplasmic bodies in the form of lipid droplets and membrane bound dense bodies

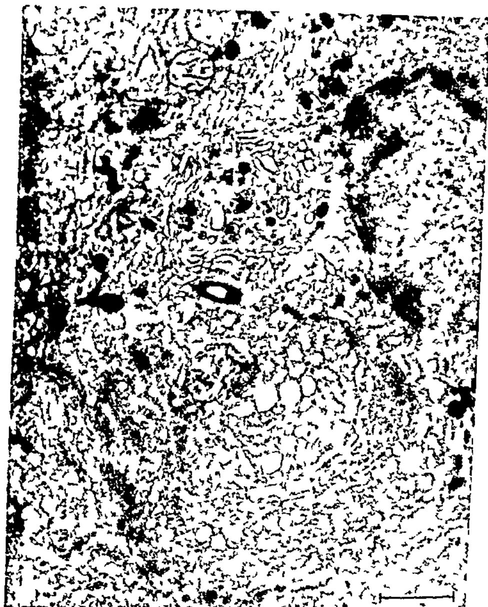


Fig 4
LTH cell with a *Nebenkern* formation The cytoplasm contains oval or irregularly shaped secretory granules × 18 000

jected to radiothyroidectomy and continuous oestrone treatment but no morphological study of the pituitary glands was performed

It appears from both the light and electron microscopic pictures in this study that the two described predominating cell types show the morphological characteristics of thyroidectomy cells (*Barnes* 1963 *Cardell* 1964 *Lundin & Schelin* 1964) and LTH cells (*Barnes* 1963) respec

lively. The proliferating cells have the same morphological appearance as those in the highly differentiated primary tumours induced by radiothyroidectomy (*Lundin & Schelin* 1964, *Schelin & Lundin* 1968) and oestrogen treatment (*Vellkamp & Kwa* 1966). The results suggest that two cell types can proliferate simultaneously under the influence of hormonal derangement.

Based on extensive studies on experimental pituitary tumours Furth and collaborators (*cf. Furth & Clifton* 1966) have suggested that the pituitary gland is "a mosaic of different functional cell types independently regulating specific functions". The different cell types control their target organs and are controlled by stimuli from these target organs. Disruption of these controls may be followed by hyperfunction and hypertrophy of the pituitary cell type involved which eventually may lead to tumour formation. This idea has been questioned by *Kwa* (1961) in his study of experimental pituitary tumours. In mice of the C57 Bl strain he found a close parallelism between pituitary tumours induced by radiothyroidectomy and tumours induced by oestrone treatment as regards incidence and rate of growth and thought that between 10 and 50 per cent of the hypophyseal cells were involved in the tumour formation. Kwa's results suggest that the mouse pituitary gland contains undifferentiated cells and that these cells take part in the tumour formation and differentiate according to the prevailing hormonal state of the tumour bearing animal. In a later report *Clifton* (1966), however, using tritiated thymidine in a study of cell population kinetics during induction of thyrotrophic tumours, concluded that thyrotrophic tumours arise from cells that initially constitute a relatively small percentage of the anterior pituitary cell population and that all or many of the remaining pituitary cells persist during tumour induction.

It is possible that a simultaneous proliferation occurs from existing TSH and LTH cells, but the results of this study do not contradict the possibility that the pituitary gland contains pluripotent cells capable of differentiation into TSH and LTH cells under the influence of the hormonal derangement. The results from other studies speak in favour of the latter theory. In an earlier study of Furth's tumour MtT F4 (*Furth et al.* 1956) we have suggested the existence of pluripotent cells in the rat pituitary (*Schelin et al.* 1964) and more recent results obtained by *Vellkamp & Kwa* (1966) indicate that oestrogen induced tumours can change their morphology if the hormonal imbalance is changed from an oestrogen excess into thyroid hormone deficiency by radiothyroidectomy.

Tumour induction by combined radiothyroidectomy and continuous oestrogen administration as well as hormone treatment of dependent and autonomous tumours induced by radiothyroidectomy or oestrogen treatment may perhaps add some information about this problem. Such experiments are in progress.

The purpose of the present study was to discuss the features outlined above and to report on a family in whom transmission of a D/D type translocation has been found through at least three generations. In this family one child *with* a D/D translocation and multiple abnormalities was born and also a child *without* D/D translocation but with Down's syndrome whose father was carrier of the translocation.

MATERIAL AND METHODS

The propositus in this study was a newborn boy who was referred for chromosome study a few weeks after birth because Patau's syndrome was suspected.

Case History

The patient (Fig. 1, III 4) was born in March 1968 in a local hospital. Towards the end of the pregnancy the mother suffered from high blood pressure and oedema and was treated with Rontyl®. The delivery was prolonged. The birth weight of the child was 2 400 grammes. At the age of two weeks the infant was transferred to the Cardiac Clinic at Queen Louise's Children's Hospital in Copenhagen to be examined for congenital heart disease and various malformations.

The shape of the skull was somewhat pointed anteriorly, the forehead was high, fontanelles normal. The ears were low set, the eyebrows met, the nose was lumpy, the distance between nose and mouth was large and the chin receded. The child had a bilateral cleft palate involving both the hard and the soft palate. The chest bulged and a loud systolic murmur was heard. The testes could not be felt, and the prepuce was split. The hands and feet were clumsy with syndactylized third and fourth toes on the right foot, abnormal position of the thumbs and mongolian creases in the palms.

Chest X-ray revealed enlarged heart, increased peripheral vascular markings and presumably a vascular ring around the oesophagus. Furthermore bilateral brachymetacarpia and syndactyly of the third and fourth toe of the right foot was seen.

Cardiac catheterization and angiocardiography showed atrial septal defect with left-right shunt, ventricular septal defect, valvular pulmonary stenosis and right arch of the aorta.

Chromosome analysis of peripheral blood: D/D translocation—see Fig. 2.

The infant had to be fed by tube and was returned to the local hospital. Subsequently he was transferred to a special hospital for children with malformations. —His present condition is extremely poor and occasional attacks of cyanosis occur.

After the D/D translocation was discovered, examination of the whole family was carried out (Fig. 1).

There was a strong reluctance to this examination, not least because the family quite deliberately tried to "shield" the child III 1, knowing that it was retarded. Eventually a blood sample from the child was obtained but a more detailed physical examination was not permitted. Inter alia we were not allowed to inspect the palms. However, the appearance of the child was typic[illegible] m[illegible] [illegible] it could not walk or talk at the time of examination at the age of 1 month.

Apart from these two children, all other members of the family seemed to be intelligent, to all appearances they were physically well equipped and without any known malformations. II 4 was born out of wedlock and was a half-sister of II 5–10.

All miscarriages were spontaneous: II 1, 4 months, II 5, 3 months, II 8, 3 months. II 6 died at the age of 3 years from scarlet fever and was [illegible] completely normal.

RESULTS

Chromosome studies have been carried out using the *normal technique* on peripheral blood which was cultivated for 48 hours. Seventeen members of the family were investigated, the study being carried *out retrogradely* from III 4 and so far back as possible. The appearance of

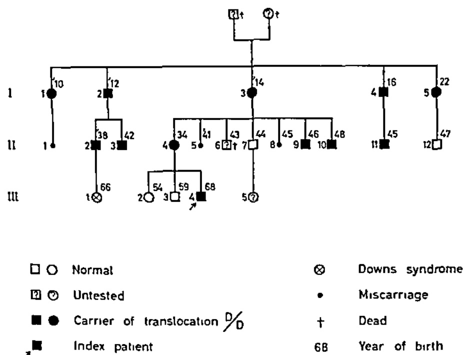


Fig 1
Pedigree of family

the translocation in three generations has been mapped out as appears from Fig 1 Because of a negative attitude of the family and because of geographical conditions we had to abstain from examining spouses of the members of the family with the exception of the father of the propositus who had a normal karyotype —In all cases complete analysis of 20 cells was carried out As will be seen 12 persons with the D/D translocation were found In these subjects no signs were revealed of mosaic evaluated on the basis of the blood cultures Furthermore there was no increase in the number of aneuploid cells no other structural chromosome aberrations and no abnormal number of chromatid aberrations

On the basis of a number of optimum cells we tried to evaluate which of the 6 chromosomes in the 13 15 group were part of the translocation chromosome In this connexion it can be established that one of the chromosomes in the 13 15 group is larger than the remaining three and that presumably one chromosome is smaller Furthermore the two sets of arms in the translocation chromosome show a distinct difference in size The translocation chromosome in most of the cells resembles something between a chromosome No 2 and No 3 Hence it is most

	Orig nal	Translocat on	Gametes 1	Gametes 2 normal	Zygotes	
Homologous chr		Presumed lost				Trisomy Transloc.
						Monosomy
Heterologous chr		Presumed lost				45 Transloc
						46 Normal
						Trisomy Transloc
						Monosomy
						Trisomy Transloc
						Monosomy

Fig 5
Diagramme showing the results of a homologous and non homologous D/D type translocation

likely a 13/15 translocation although a 13/14 translocation cannot be excluded

As mentioned above the child III 1 showed clinical signs of mongolism In this case also 20 cells were analyzed all of which *showed regular* 21 trisomy without other aberrations (Fig 3)

DISCUSSION

Characteristics and Frequency of the Translocation

On the basis of purely morphological studies the translocation chromosome appearing in this family seems likely to involve one chromosome No 13 and one No 15 *Hamerton et al* (7) reached the same result in the families examined by them and demonstrated it in Fig 4 of their report Studies of karyotypes in our material seem to correspond completely to these findings No DNA replication analyses were made but such analyses have been planned in connexion with coupling analyses and enzymatic studies at a later time

As shown diagrammatically in previous studies among others by *Hamerton et al* (7) and *Stahl et al* (15) and as mentioned by several other authors on the basis of theoretical considerations it must be expected that translocation of two homologous chromosomes would give rise only to monosomies which are not viable and to trisomies with the translocation chromosome (Fig 4) Translocation between two non homologous chromosomes will also result in monosomies and trisomies with the translocation chromosome Furthermore normal persons and cases having the translocation chromosome but without other aberrations will appear these would occur in the ratio of 1 1 (Fig 4)

The reason why trisomies did not occur in the present family can supposedly be gametic selection or early death in utero It is difficult to assert whether the number of miscarriages in this family is unusually high No information regarding miscarriages on the I plane in Fig 1 is available Among those who could get the translocation in this family there are 12 members with and five without which is not statistically significantly different from the ratio of 1 1 the theoretically expected ratio which has been found also in other large families *e g* by *Walker & Harris* (20) and *Hamerton et al* (7)

As appears from Fig 1 transmission of the translocation through three generations has been demonstrated However with a greater number of carriers in the I generation it must be presumed that one of the great grandparents of the propositus has also been carrier of the translocation and hence there is evidence for the transmission through four generations

A The characteristic mentioned in the introduction that many healthy carriers are found in the large families with D/D translocations on whom reports are available seems to indicate that the material lost by the translocation is of no genetically significant importance in these cases Consequently it must be envisaged that a not insignificant number of families with this type of translocation *may* be found which will not be known till mechanical techniques make possible analyses of large groups of population

	Original	Translocation	Gametes 1	Gametes 2 normal	Zygotes	
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						Monoso[illegible]

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father was carrier of the D/D translocation —A possible relationship between the translocation and the congenital malformations is discussed emphasizing that the cases of D/D translocation with congenital abnormalities described in the literature have not presented the same abnormalities — A possible predisposition to non disjunction is discussed on the basis of the discovery of the mongol child and of cases reported in the literature of mongolism and other syndromes with specific chromosome abnormalities

Finally reference is made to observation of development of malignancy in the presence of chromosome aberrations of various genesis

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Acta path. microbiol. scand. 75: 555–568, 1969

The University Department of Pathology, General Hospital,
Malmö, Sweden

FATAL NECROTIZING LESIONS OF UNCERTAIN AETIOLOGY

By

F. Linell and G. Östberg

Received 8.x.68

In the course of a few months we observed two clinically and pathologically strikingly similar cases with peculiar lesions. These are the only two cases of this type we have ever seen and, as far as we know, no such cases have been published before. Common to both cases was a long history of intermittent fever and diffuse abdominal symptoms. Necropsy showed widespread necrotic foci with a sparse limiting inflammatory reaction in the mesentery of the small intestine and small necrosis and ulceration in the intestinal wall. One of the patients had widespread necrotic foci in the lungs and kidneys and the other had disseminated foci in the liver, spleen and bone marrow. Neither histological examination nor culture revealed any infectious agent. The cause of the changes is obscure. A brief preliminary report was published (*Linell et al.* 1966) and the cases are now described in detail to draw attention to the lesions in the hope that it will lead to the publication of further cases—which have presumably occurred also in other places—and provide more knowledge of the condition.

CASE REPORTS

Case 1 (autopsy 965/63)

At the end of 1962 a 57 year old, previously healthy janitor without any known hereditary disease began to have bouts of fever about once a fortnight. Temperature peaks of 40° C occurred and persisted for a day or so. A few months later he began to have such spells of fever about once a week. His general condition was astonishingly good and he could manage his work between the attacks of fever. He lost about 0 kg of bodyweight within 4 months, but he did not feel short of breath and he had no cough. He sought medical advice on April 16, 1963, when fluoroscopy of the chest revealed fairly well outlined densities the size of hens' eggs. The E.S.R. (Westergren) was 43 mm/1 hour. The patient was admitted to the hospital, where he remained until death on September 15, 1963. He was in a relatively good general condition and physical examination revealed nothing remarkable. Fever peaks (initially observed about once a week) soon occurred daily. During the last few weeks before death body temperature was constantly at 38–39° C. The patient became weaker and weaker, he continued to loss weight and sometimes had diarrhoea. Chest x-ray showed progressive pulmonary changes with large rounded densities. Other roentgen examinations (urinary tract, oesophagus, throat, stomach, duodenum, colon, skeleton, gallbladder) revealed nothing remarkable.

Bronchoscopy showed no abnormality.

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Figs 1–3

Fig 1 Case 1 The l· ngs with large white infiltrat s Note vessels and bronchi inside the necrosis

Fig ⁹ Case 1 Large ellow v hite mas n the mesent ry

Fig 3 Case 1 Multiple yellow white tum ur like infiltrations in the ¹

and no primary glomerular changes The renal parenchyma between the lesions was of normal appearance Most of the foci showed no cellular reaction but a few contained interstitial fairly dense infiltrates of round cells and macrophages (Fig 10) *The e infiltrates were inconstant and not dominating the picture*
Staining with Gram stain Ziehl Neelsen stain and various fungus stains (Gridley Grocott silver methenamine) proved negative

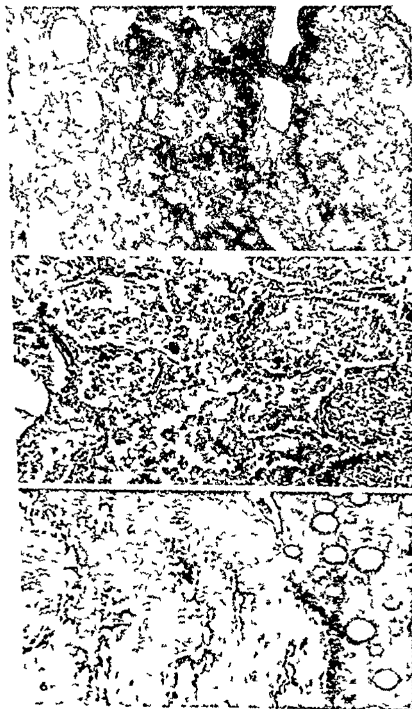
Case 3 (autopsy 603/64)

In June 1963 a 43 year-old menopausal female clerk who had always felt well except for periods of mild anaemia which responded to iron therapy and who had no known hereditary disease began to have diffuse dull abdominal pain Her appetite was poor and in a few months she lost about 10 kg At the end of 1963 the abdominal pain subsided but in the beginning of 1964 the patient had increasing anaemia and was abnormally tired She was admitted to the hospital on April 1 1964 During the first 4 weeks in hospital she had fever with peaks of 38 –40 C. A lump in the abdomen was palpated and surgical exploration on May 19 revealed that the lymph nodes in the radix mesenterii were markedly enlarged The liver was enlarged Histological examination of a biopsy specimen of the mesentery showed only fatty tissue necroses
In the further course signs of fibrinolysis and intestinal bleeding appeared. Clouding and neck stiffness developed and the patient died on June 1 1964
Roentgen examination showed soft tissue densities in the abdomen but otherwise nothing remarkable
Laboratory studies Anaemia with Hb of 37 per cent (5.5 g/100 ml) and R B C. 1.9 mill W B C. about 2 000 Diff count neutrophil granulocytes 80 90 per cent lymphocytes and monocytes 10 1 per cent No eosinophilia E.S R. (Westergren) on different occasions 28–5 mm (fibrinolysis) 1 h All other tests were essentially normal
Electrophoresis Slightly increased α_1 and α_2 No increase of γ fraction
Bacteriological studies Repeated culture of the blood gave no growth Culture of C.S F and urine was negative
Serological studies negative (also Wassermann reaction)
Therapy Tetradecin penicillin streptomycin without any demonstrable effect Prednisone 1 week in April without any effect
Autopsy A fairly lean middle aged woman *Bluish red discoloration of the skin* over the sacrum All serous cavities contained clear fluid (pericardium 300 ml right pleura 250 ml left pleura 300 ml peritoneum 300 ml) and had shiny surfaces. The cardiovascular system showed nothing remarkable The lungs were moderately oedematous and showed small areas of atelectasis
The liver was enlarged (2 210 g) with flamy markings and scattered yellow punctate foci Similar yellow foci were found in the spleen which was slightly enlarged (290 g) (Fig 11)
The mesentery of the ileum contained a 5 cm thick polycyclic limited yellow white *infiltrate that resembled a large necrotic focus (Fig 12) In the corresponding* segment of the ileum was a 1 cm wide semicircumferential ulceration –The colon was full of clear red blood Retroperitoneally and around the pancreas were enlarged lymph nodes and yellow white foci *The vertebral marrow* contained a few diffusely outlined grey white foci
Over the right cerebral hemisphere were 5 mm thick red brown masses *of old* blood adherent to the inner surface of the dura Other organs examined were of gross normal appearance

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Figs 4–6

Fig 4 Case 1 Lung with large necrosis to the right The alveolar structures and bronchi and vessels can be seen in the necrosis The adjacent preserved lung tissue (to the left) shows some macrophages in the *alveoli* × 25
Fig 5 Case 1 Lung necrosis The alveoli filled with granular amorphic masses In the walls nuclear remnants × 120
Fig 6 Case 1 Necrotic mesentery with granular material *Some endothelial cell* preserved × 120



and no primary glomerular changes. The renal parenchyma between the lesions was of normal appearance. Most of the foci showed no cellular reaction but a few contained interstitial fairly dense infiltrates of round cells and macrophages (Fig. 10). These infiltrates were inconstant and not dominating the picture.

Staining with Gram stain, Ziehl Neelsen stain and various fungus stains (Friedly Gomori silver methenamine) proved negative.

Case 2 (autopsy 603/64)

In June 1963 a 49 year old menopausal female clerk who had always felt well except for periods of mild anaemia which responded to iron therapy and who had no known hereditary disease began to have diffuse dull abdominal pain. Her appetite was poor and in a few months she lost about 10 kg. At the end of 1963 the abdominal pain subsided but in the beginning of 1964 the patient had increasing anaemia and was abnormally tired. She was admitted to the hospital on April 1 1964. During the first 4 weeks in hospital she had fever with peaks of 38–40 C. A lump in the abdomen was palpated and surgical exploration on May 12 revealed that the lymph nodes in the radix mesenterii were markedly enlarged. The liver was enlarged. Histological examination of a biopsy specimen of the mesentery showed only fatty tissue necroses.

In the further course signs of fibrinolysis and intestinal bleeding appeared. Clouding and neck stiffness developed and the patient died on June 1 1964.

Roentgen examination showed soft tissue densities in the abdomen but otherwise nothing remarkable.

Laboratory studies. Anaemia with Hb 37 per cent (5.5 g/100 ml) and R.B.C. 1.9 mill. W.B.C. about 2000. Diff. count: neutrophil granulocytes 80–90 per cent, lymphocytes and monocytes 10–15 per cent. No eosinophilia. E.S.R. (Westergren) on different occasions 25–5 mm (fibrinolysis) 1 h. All other tests were essentially normal.

Electrophoresis. Slightly increased α_2 and α. No increase of γ fraction.

Bacteriological studies. Repeated culture of the blood gave no growth. Culture of C.S.F. and urine was negative.

Serological studies negative (also Wassermann reaction).

Therapy. Tetradecin penicillin streptomycin without any demonstrable effect. Prednisone 1 week in April without any effect.

Autopsy. A fairly lean middle aged woman. Bluish red discoloration of the skin over the sacrum. All serous cavities contained clear fluid (pericardium 350 ml, right pleura 250 ml, left pleura 300 ml, peritoneum 300 ml) and had shiny surfaces. The cardiovascular system showed nothing remarkable. The lungs were moderately oedematous and showed small areas of atelectasis.

The liver was enlarged (2210 g) with flamy markings and scattered yellow punctate foci. Similar yellow foci were found in the spleen which was slightly enlarged (280 g) (Fig. 11).

The mesentery of the ileum contained a 5 cm thick polycyclic limited yellow white infiltrate that resembled a large necrotic focus (Fig. 12). In the corresponding segment of the ileum was a 1 cm wide semicircumferential ulceration. The colon was full of clear red blood. Retroperitoneally and around the pancreas were enlarged lymph nodes and yellow white foci. The vertebral marrow contained a few diffusely outlined grey white foci.

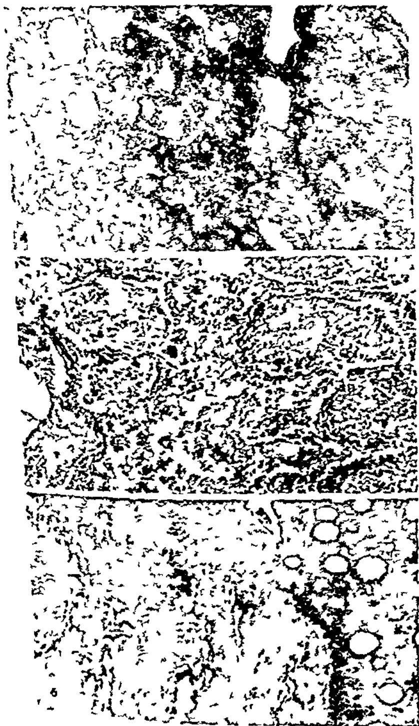
Over the right cerebral hemisphere were 5 mm thick red brown masses of old blood adherent to the inner surface of the dura. Other organs examined were of gross normal appearance.

Figs. 4–6

Fig. 4. Case 1. Lung with large necrosis to the right. The alveolar structures and bronchi and vessels can be seen in the necrosis. The adjacent preserved lung tissue (to the left) shows some macrophages in the alveoli. × 25.

Fig. 5. Case 1. Lungnecrosis. The alveoli filled with granular eosinophilic masses. In the walls nuclear remnants. × 120.

Fig. 6. Case 1. Necrotic mesentery with granular material. Some endothelial cells preserved. × 120.









Micros opy The lungs myocardium and adrenals contained discrete groups of staphylococci in some areas of the lungs and myocardium there was a mild leucocytic reaction

The mesentery and omentum contained widespread granular eosinophilic necrotic foci (Fig 13) where fatty tissue and vascular structures were being destroyed by the necrosis Only endothelial cells were preserved and scattered histiocytes bordered the necrotic foci The intestinal lesion consisted of a necrotic area not surrounded by a limiting inflammatory reaction and was continuous with mesenteral necroses (Fig 14) The lymph nodes were partly or entirely necrotic The necroses appeared to begin in the marginal sinus while some of the follicles were preserved Similar changes of the lymph nodes were seen in the retroperitoneum and in the left supraclavicular fossa Other lymph nodes (mediastinum axillae groins right supraclavicular fossa) were of normal appearance

The acinar structure of the liver was preserved with numerous scattered necroses (Fig 15) Sometimes they were centro acinar but most often irregularly arranged and involving several acini In the necroses which were granular and slightly eosinophilic the outlines of the liver cells and fragments of nuclei were discernible No vascular changes no cellular infiltrates (Fig 16) The spleen showed large eosinophilic necrotic foci without any limiting inflammatory reaction (Fig 17) In both the vertebral and the femoral marrow were patchy necrotic areas without any inflammatory reaction (Fig 18)

Culture of specimens from the mesentery gave growth of coliform rods staphylococci and enterococci Culture for fungi was negative Guinea pig test and culture on Loewenstein s medium were negative Staining for bacteria and fungi was negative

DISCUSSION

Clinically and pathologically the two cases showed a very similar and peculiar picture We have never seen such cases before and we have not been able to trace any in the literature Various differential diagnoses might be considered

The clinical picture with periodic fever as the most prominent symptom might point to malignant lymphogranulomatosis (Hodgkin s disease) but the anatomical findings lent no support to this possibility The most striking changes were large necrotic foci with very sparse inflammatory reaction Such lesions can be seen in Hodgkin s disease but at least when no specific treatment has been given there are usually coexisting typical changes No such changes were observed in our cases despite extensive histological examination The distribution of the lesions also differed from that usually seen in Hodgkin s disease Involvement of the mesenteric lymph nodes appeared to be only one component of a more extensive necrosis of fatty tissue The lymph nodes in the retroperitoneum and in case 2 in the left supraclavicular fossa also showed necrosis while all the other lymph nodes appeared

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Figs 7-9

Fig 7 Case 1 Retroperitoneal lymphnode with large granular necrosis without cellular reaction To the right some preserved lymphocytes × 120

Fig 8 Case 1 Kidney To the left large necrosis Some glomeruli are preserved To the right normal kidney tissue × 25

Fig 9 Case 1 Kidney necrosis The tubules are necrotic A few nuclear remnants in the granular masses interstitially Glomerulus normal × 1?0


13

times been discussed under the heading of a so called visceral type of *relapsing febrile nonsuppurative panniculitis* (visceral Weber Christian syndrome) (*Harrington et al* 1961 *Mostoft et al* 1947 *Steinberg* 1953 *Ungar* 1946) Judging from published autopsies the condition is characterized by co existing subcutaneous changes of the type seen in Weber Christian s disease Lesions of the abdominal fatty tissue showed different stages of inflammation ranging from acute cellular inflammation and lipogranulomatous changes to fibrosis None of these published cases appear to have shown such necrosis as in our cases or manifestations in the lungs liver or kidneys for example

The veterinary medical literature (*Hoflund et al* 1953) contains reports according to which cattle presented fatty tissue necroses in the mesentery large and small conglomerates along the duodenum small intestine and occasionally along the rectum They are well outlined first yellow white and then mottled In the chronic form they are limited by pronounced fibrosis with abundant connective tissue round the empty spaces after dissolved fat The changes are thought to be secondary to some other abdominal disease such as traumatic peritonitis or enteritis The actual nature of these lesions is obscure The lesions do not resemble the necrosis found in our cases

Some 20 cases have been published under the name of *progressive arterial occlusive disease* (Kohlmeier Degos) Most of them have been seen in young men with patchy atrophy of the skin and multiple perforations of the small intestine (*Strole et al* 1967) Necroses in other organs have also been reported The cause of the disease appears to be obliterating fibrous endarteritis and this typical vascular change which was not seen in our cases also excludes this diagnosis

The lung lesions in our case 1 might at first be thought to resemble *proteinosis alveolaris pulmonum* (*Rosen et al* 1958) But they were not PAS positive and they had no acicular spaces No other visceral manifestations are known in proteinosis alveolaris and this diagnosis may therefore be ruled out in our cases

Summing up a search of the literature failed to reveal any analogous cases and at least for the time being our cases appear to be examples of a previously unknown disease Similar cases have probably been observed in other places but either not published or reported under such names that we could not trace them

The cause of the v ... pre d changes in our cases is unknown The presence of intestinal ne ro es ar l widespread mesenteric lesions in

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Figs 13–1

Fig 13 C e ? Granular necr sis in the omentum Vessel with thin walls and partly preser e l endothelial c lls × 1 0

Fig 14 Case 2 Necrosi of the intestinal wall × 4

Fig 15 Case 2 Large irregular li ernecrose without cell ilar reaction × 25

Steinberg B Systemic nodular panniculitis Amer J Path 29 1059 1081 1953
Strole W E Clark W H & Isselbacher K J Progressive arterial occlusive disease (Köhlmeier Degos) New Engl J Med 276 195 201 1967
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Ungar H Relapsing febrile nodular inflammation of adipose tissue (Weber Christian syndrome) J Path Bact 58 175-185 1946
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Wegener F Pneumogene allgemeine Granulomatose (PG) – Sog Wegenersche Granulomatose Lehrbuch der spez pathol Anatomie von Kaufmann Staemmler (Ergänzungsband I) Walter de Gruyter & Co Berlin 1967 P 225-229

Acta path microbiol scand 75 569–587 1969

The Department of Pathology Karolinska sjukhuset Stockholm Sweden

THE INTRARENAL VASCULAR ALTERATIONS IN EXPERIMENTAL CHRONIC PYELONEPHRITIS AND THEIR RELATIONSHIP TO THE DEVELOPMENT OF HYPERTENSION

By

ARNE LJUNGQVIST and JOHN RICHARDSON

Received 16 x 68

The development of hypertension in human subjects with chronic pyelonephritis is generally ascribed to the lesions of the intrarenal artery walls which lead to a form of renovascular hypertension It has been noted however that there are normotensive cases with chronic pyelonephritis and severe changes in the walls of the intrarenal vessels as well as cases with hypertension in which the vessel wall changes are relatively mild (*Brod* 1957 *Kleeman Hewitt & Guze* 1960 *Lagergren & Ljungqvist* 1962) Moreover *Heptinstall* (1962) found no relationship between pyelonephritic damage to the walls of the intrarenal vessels and the occurrence of hypertension in rats

In a previous study of the vasculature of the human pyelonephritic kidney glomerular damage resulted in the formation of aglomerular juxtamedullary arterioles leading to the medulla whereas the cortical arteriole glomerular units degenerated and disappeared (*Lagergren & Ljungqvist* 1962) The alterations were considered to form a possible morphological basis for the development of cortical ischaemia and thus hypertension by enhancing the medullary blood flow and impeding that of the cortex In the human material however it could not be ascertained that the alterations in the intrarenal vasculature had actually preceeded any blood pressure elevation Experimental data rather suggest that glomerular alterations develop secondarily to hypertension (*Heptinstall & Hill* 1967)

To investigate the pathogenetic role of alterations in the intrarenal micro-angioarchitecture in the development of hypertension in chronic pyelonephritis the following experiments were carried out in which the intrarenal vascular alterations in normotensive and hypertensive rats

This study was supported by the Swedish Medical Research Council (project No B68 12X 716 03) the *Swedish National Association against Heart and Chest Diseases* and the *Swedish Life Insurance Companies*

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is desired In the present study sublimate was excluded from the original Helly s solution since its radiopacity rendered the micro angiograms useless The modified fixative then consisted of Zenker s solution devoid of sublimate and 10 per cent neutral formalin in equal parts

A few rats did spontaneousl some time after hypertension had developed the other rats were killed by injection of Micropaque during ether anaesthesia after normotensive and hypertensive periods of varioiu lenghts In all rats a thoracotomy was performed with one cannula inserted into the thoracic aorta downwards to a level just above the origin of the right renal artery another cannula being inserted into the aorta but placed towards the heart The cannulae were attached to a bottle containing a 10 per cent water suspension of Micropaque This was forced into the vessels of the rat by the method described above for the previously removed kidneys During this injection the living anaesthetized rats died presumably from widespread embolic occlusion of the vascular system by barium particles The hearts were injected in order to study the myocardial vasculature in hypertension and this will b the subject of a separate report The kidneys were longitudinally sectioned and fixed in the modified Helly s solution for 24 hours

After fixation the kidneys were embedded and processed for stereomicro angiography according to previously described procedures (*Ljungqvist* 1963) The micro angiographed blocks were re embedded and thin sections cut and stained for histological examinations Blocks from some kidneys from control rats and from pyelonephritic rats with and without hypertension were serially sectioned to make it possible to trace the micro angiographically visualized vessels through the renal tissue The degree of juxtaglomerular granulation was determined in each kidney by calculating the juxtaglomerular index (JGI) according to *Hartroft & Hartroft* (1953) For this all glomeruli in an entire frontal section were counted

The twelve hypertensive rats were tentatively divided into two equal groups in *one group the rats hypertension had been present for only a brief period (1–3* weeks) whereas in the other group the rats had been hypertensive for periods varying between 6 and 15 weeks This was done in order to see whether the duration of the hypertension would influence the morphological picture of the infected kidney but since no such difference between the two groups was observed all the hypertensive rats will be presented and discussed as one group

RESULTS

Blood Pressure and JGI (Table 1)

E coli—injected animals The mean blood pressure of these rats was 115 mm Hg before the beginning of the experiments the highest reading being 135 mm Hg in two of the rats each at only one measurement All animals remained normotensive as long as the left presumably non infected kidney was present Following removal of this kidney 10 weeks *after injection of bacteria in 9 rats and 18 weeks after the* injection in the remaining 30 rats hypertension developed in 12 rats after 2½ to 8 weeks The latent period for the development of hypertension after removal of the left kidney did not differ between rats in which the nephrectomy was done 10 weeks after the injection and rats in which it was done 18 weeks after the injection

In the 12 rats that developed hypertension the blood pressure became established well above 150 mm Hg and remained there until the death of the animals These 12 rats all showed severe chronic pyelonephritis of the right kidney (see below) In the 23 normotensive rats no blood pressure reading above 13 mm Hg was recorded In eight of these rats either no evidence of inflammation was seen in the right kidney or there were minimal inflammatory changes observed

in the pelvic area These eight rats were regarded as having not acquired pyelonephritis In the remaining 15 *normotensive rats there* was chronic pyelonephritis of a varying degree in the right kidney (see below)

There were significant differences between the *JGI values of the* right and left kidneys in both the hypertensive and normotensive rats with pyelonephritis As is seen in Table 1 these differences are due *to a lowering of the JGI of the infected kidneys*

TABLE 1

Blood Pressure and JGI (Juxtaglomerular Index) in Hypertensive and Normotensive Rats with Chronic Pyelonephritis in the Right Kidney in Rats Where Inoculation of Bacteria Failed to Result in Pyelonephritis and in Control Rats Given Saline

Group of rats	No of rats	B p¹	B p²	B p³	JGI (mean ± SE)	
					Left	Right
Hypertens.	12	115	170	175	16.5 ± 1.4	1.8 ± 0.4
Normotens (p n)§	15	115	115	115	19.1 ± 1.8	9.6 ± 2.2
Normotens (no p n)	9	115	110	120	20.2 ± 1.9	19.8 ± 3.2
Controls	10	120	120	115	21.5 ± 1.1	23.2 ± 2.1

B p¹ mean blood pressure level at start of experiment
B p² mean blood pressure at left nephrectomy
B p³ mean blood pressure at death of animals
p n = pyelonephritis
‡ difference in JGI between right and left P < 0.01
§ difference in JGI between right and left P < 0.001

Control animals The mean blood pressure of these rats was *120* mm Hg before the beginning of the experiments and 115 mm Hg when the animals were sacrificed No reading above 135 mm Hg was *recorded* in these animals on any occasion *The* difference in JGI between the left and right kidney was statistically not significant

Microscopic Features

1 *Pyelonephritic hypertensive rats* The pyelonephritic *lesions* in the right kidney of these rats varied slightly in extent from one rat to another *In affected areas the lesions* were histologically similar and consisted of cortical tubule atrophy increase in *interstitial* connective tissue and crowding of the glomeruli In the interstitial tissue *an inflammatory cell infiltration* was evident The glomeruli in areas of pyelonephritic lesions were *of* variable sizes most being smaller than normal In some slight fibrosis of Bowman s capsule was seen Some *glomeruli in the affected* areas and many *of the glomeruli in the* minimally affected and unaffected areas showed conspicuous changes in the glomerular capillary tufts These changes consisted of capillary wall thickening which in some instances led to obliteration of the capillary lumen (Figs *1B* and 3B) The thickened capillary walls often gave

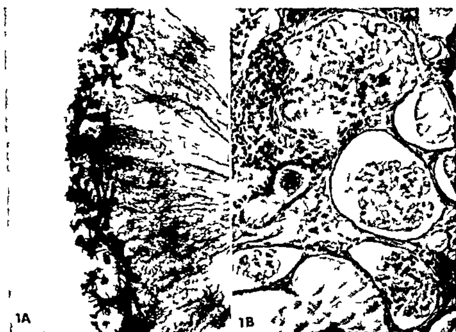


Fig 1

A Micro angiogram of a pyelonephritic kidney from a hypertensive rat The cortex is thinned and its arteries kinked and spiralled Only a few glomeruli are visualized There is hardly any visualization of the postglomerular vasculature in the cortex whereas there is complete visualization of the m dullary ves els Many of these derive from aglomerular juxtamedullary arterioles Cf Fig 5 A × 1?

B Histological section from the cortex of the kidney in Fig 1A Theres is inflammation fibrosis and some distension of the tubules which contain protein like material The two glomeruli in bottom part of picture are small the glomerulus in centre shows partial occlusion of its capillary tuft the glomerulus in top of picture is severly altered by complete occlu ion of its capillary tuft and there is thickening of the wall of the afferent arteriole at the vascular pole van Gieson × 180

a positive reaction with fibrin stains and in some areas this was pronounced giving the appearance of fibrinoid necrosis A similar fibrinoid material was seen in some of the afferent arteriole walls (Fig 1B) In many glomerular capillaries and in some extraglomerular vessels structureless particles that stained like fibrin were also encountered in the lumina The tubules in less affected areas were often dilated with atrophy of the epithelium and casts in the lumen Arteriolosclerosis was rare

In 9 of the pyelonephritic kidneys the inflammation and atrophy in the cortex was extensive although areas of cortical tissue were left unaffected by tubular atrophy interstitial reaction and glomerular crowding In the remaining three rats the lesions were clearly patchy In the former 9 kidneys the medulla also showed widespread changes consisting of moderate interstitial reaction with fibrosis and infiltration by inflammatory cells The most conspicuous alteration in the medulla was the presence of both collapsed and dilated tubules the latter often

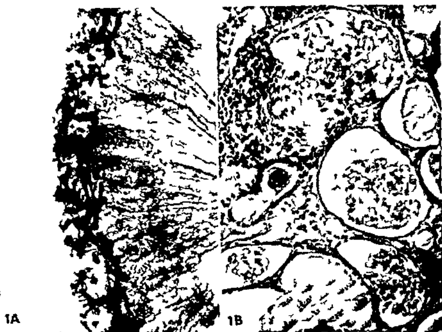


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Group of rats	No of rats	B p[1]	B p[2]	B p[3]	JGI (mean ± SE)	
					Left	Right
Hypertens.	12	115	120	175	16 5 ± 1 4	1 8 ± 0 4
Normotens. (p n) §	15	115	115	115	19 1 ± 1 8	9 6 ± 2 2
Normotens (no p n)	8	115	110	120	20 2 ± 1 9	19 8 ± 3 2
Controls	10	120	120	115	21 5 ± 1 1	23 2 ± 2 1

B p[1] mean blood pressure level at start of experiment
B p[2] mean blood pressure at left nephrectomy
B p[3] mean blood pressure at death of animals
p n = pyelonephritis
difference in JGI between right and left $P < 0\ 01$
§ difference in JGI between right and left $P < 0\ 001$

Control animals The mean blood pressure of these rats was 120 mm Hg before the beginning of the experiments and 115 mm Hg when the animals were sacrificed No reading above 135 mm Hg was recorded in these animals on any occasion The difference in JGI between the left and right kidney was statistically not significant

Microscopic Features

Pyelonephritic hypertensive rats The pyelonephritic lesions in the right kidney of these rats varied slightly in extent from one rat to another In affected areas the lesions were histologically similar and consisted of cortical tubule atrophy increase in interstitial connective tissue and crowding of the glomeruli In the interstitial tissue an inflammatory cell infiltration was evident The glomeruli in areas of pyelonephritic lesions were of variable sizes most being smaller than normal In some slight fibrosis of Bowman s capsule was seen Some glomeruli in the affected areas and many of the glomeruli in the minimally affected and unaffected areas showed conspicuous changes in the glomerular capillary tufts These changes consisted of capillary wall thickening which in some instances led to obliteration of the capillary lumen (Figs 1B and 3B) The thickened capillary walls often gave

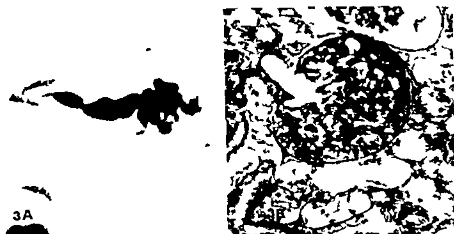


Fig 3

A Micro angiogram from fairly unaffected kidney tissue in a pyelonephritic kidney of a hypertensive rat An afferent arteriole is seen to lead into an incompletely filled glomerular tuft There is no evidence of filling of the postglomerular vasculature. × 160

B Histological section from the same tissue as in Fig 3A showing the incompletely filled glomerulus in which there is thickening of the capillary walls and partial occlusion of the lumina Patches of fibrinoid change are seen in the tuft Ladewig × 160

affected and the less affected areas there were numerous glomeruli which were only partially visualized and showed no or poor filling of the postglomerular vessels (Fig 3A) In the histological sections these glomeruli showed the changes of partial or complete occlusion of the capillary tuft fibrinoid thickening and degeneration of the capillary walls and focal fibrinoid necrosis (Fig 3B)

The good visualization of the medullary vessels was due to the fact that the glomerular alterations had not interfered with the filling of these vessels Thus in addition to normal juxtamedullary arteriole glomerular units there were a number of arterioles leading to the medulla which had an incompletely visualized and simplified glomerular tuft attached to the lateral aspect of the vessel (Fig 4A) In other juxtamedullary arterioles there was no evidence of a glomerular tuft between the site of origin of the arteriole from an interlobular or arcuate artery and its penetration into the medulla In the histological sections these two types of vessels were found to run through the vascular pole of their corresponding glomeruli which were either partially filled with contrast medium or not filled at all due to degenerative glomerular alterations (Fig 4)

The vascular pattern in the medulla of kidneys with diffuse cortical lesions was not that of normal parallel bundles of vessels with intervening capillary networks but rather a diffuse irregular and large meshed network of vessels of varying sizes (Fig 2) In medullary



Fig 4

A Micro angiogram from the juxtamedullary zone of a pyelonephritic kidney in a hypertensive rat An incompletely filled glomerular tuft is attached to an arteriole (arrow) which splits up into vessels leading to the medulla × 70

B Histological section from the same tissue as in Fig 4A showing the contrast filled arteriole which gives off glomerular capillaries as lateral twigs. The tuft is incompletely filled due to partial degeneration Ladewig × 440

areas corresponding to the most severely scarred areas in the cortex parallel bundles of abnormally thin although straight vessels were often seen (Fig 2)

The left (nonpyelonephritic) kidneys in this group of rats showed a normal micro angiographic and histological picture except for a moderate degree of pyelitis in three kidneys On closer examination however all kidneys were found to contain occasional glomeruli with patchy occlusion of the tuft fibrinoid degeneration of the capillary walls and even fibrinoid necrosis of part of the tuft In some capillaries the same type of fibrinoid material was encountered as in the opposite infected kidney

Pyelonephritic normotensive rats The histological lesions in the kidneys of these rats were quite similar to the lesions of the hypertensive rats There was a difference in the degree and extent of the lesions which was clearly true for the fibrinoid glomerular changes Although some glomeruli particularly those along the borders of pyelonephritic areas and to a certain degree also in intervening non scarred areas showed occlusion and fibrinoid necrosis of their tufts

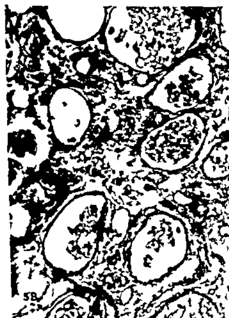


Fig

A Micro angiogram of a pyelonephritic kidney from a normotensive rat The vascular pattern is close to the normal with straight cortical vessels and good visualization of glomeruli and postglomerular vessels including the peritubular capillaries The juxtamedullary arteriole glomerular units show good filling of the glomerular tufts many of which are seen on the medullary side of the arcuate arteries Cf Fig 1A × 10

B Histological section from the cortex of the kidney in Fig 5A There is inflammation with interstitial fibrosis Some glomeruli are small but the tufts are unaffected by occlusive changes van Gieson × 165

C Histological section from another area of the kidney in Fig 5A showing one normal glomerulus (top) and one glomerulus with almost complete occlusion of the tuft (bottom) van Gieson × 165





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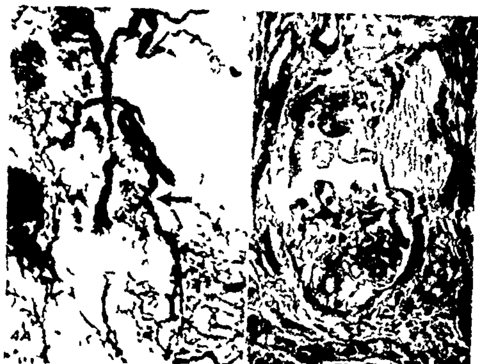


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areas corresponding to the most severely scarred areas in the cortex parallel bundles of abnormally thin although straight vessels were often seen (Fig 2)

The left (nonpyelonephritic) kidneys in this group of rats showed a normal micro angiographic and histological picture except for a moderate degree of pyelitis in three kidneys On closer examination however all kidneys were found to contain occasional glomeruli with patchy occlusion of the tuft fibrinoid degeneration of the capillary walls and even fibrinoid necrosis of part of the tuft In some capillaries the same type of fibrinoid material was encountered as in the opposite infected kidney

Pyelonephritic normotensive rats The histological lesions in the kidneys of these rats were quite similar to the lesions of the hypertensive rats There was a difference in the degree and extent of the lesions which was clearly true for the fibrinoid glomerular changes Although some glomeruli particularly those along the borders of pyelonephritic areas and to a certain degree also in intervening non scarred areas showed occlusion and fibrinoid necrosis of their tufts



Fig 3.

A Micro-angiogram of a polyomavirus kidney from a normotensive rat. The vascular pattern is close to the normal with straight cortical vessels and good visualization of glomeruli and postglomerular vessels including the peritubular capillaries. The juxtamedullary arteriole-glomerular units show good filling of the glomerular tufts, many of which are seen on the medullary side of the arcuate arteries (cf. Fig 1A) × 10.

B Histological section from the cortex of the kidney in Fig 3A. There is inflammation with interstitial fibrosis. Some glomeruli are small but the tufts are unaffected by occlusive changes. van Gieson × 165

C Histological section from another area of the kidney in Fig 3A showing one normal glomerulus (top) and one glomerulus with almost complete occlusion of the tuft (bottom). van Gieson × 165



(Fig. 3C) and fibrinoid material in the lumina of the capillaries, most glomeruli were devoid of such changes (Fig. 3B). The predominant alterations consisting of atrophy of cortical tubules, fibrosis and infiltration by inflammatory cells of the interstitial tissue and atrophy and

dilatation of medullary tubules were also less extensive and severe in these rats. The main difference between these two groups was manifest in the extent of the glomerular capillary tuft lesions which were more extensive and severe in the rats with hypertension.

By micro angiography these kidneys displayed good visualization of both the *medullary and cortical vasculatures* including the postglomerular vessels in the cortex (Fig. 5A). The pattern in pyelonephritic areas was similar to that seen in the hypertensive rats although less marked. The occurrence of aglomerular arterioles and arterioles with incompletely visualized tufts leading to the medulla was much less frequent than in the hypertensive rats. These features correspond to the fact that the degree of histological glomerular alteration was less marked in the normotensive group.

The *left presumably non infected kidney in these rats showed a* completely normal micro angiographic pattern. As in the hypertensive rats *most of these kidneys contained occasional glomeruli with thick*ened capillary walls, partial occlusion and fibrinoid necrosis of the tuft and fibrinoid material in the capillary lumina. In 3 kidneys there was pyelitis which in one case was severe, although the inflammation did not extend into the medullary or cortical tissue.

Non pyelonephritic rats. The right kidney of these rats did not show any conclusive evidence of chronic pyelonephritis. In five of these rats minimal infiltration by inflammatory cells was seen in the pelvic area. As in the control animals both the left and right kidneys showed *protein like casts in a few tubules. Occasional glomeruli in the right* kidneys displayed partial occlusion and fibrinoid change in their tufts. This feature was not encountered in the left kidneys. The micro angiographic patterns were normal in all kidneys in this group.

Control rats. The kidneys in this series of rats were morphologically similar to the non pyelonephritic rats in that the kidneys were devoid of any signs of pyelonephritis and displayed normal micro angiographic patterns. In 5 of these rats however the right kidney showed alterations in occasional glomeruli consisting in thickening of the capillary walls with occlusion of the capillary lumina and fibrinoid change.

DISCUSSION

It is well known that chronic pyelonephritis in man is associated with hypertension in a significant number of cases (*Weiss & Parker* 1939, *Kincaid Smith* 1955). The validity of the assumption that the chronic renal infection is the direct cause of the increase in blood pressure has however been questioned mainly on the basis of experimental data. According to some reports the association of the two features is a consequence of a higher susceptibility to infection of the kidney in the hypertensive animal (*Shapiro & Kobernick* 1961) and chronic pyelonephritis in the experimental animal has been reported to be unassociated with hypertension even if the kidney infection leads to

uraemia (*Shapiro & Kobernick* 1959) Quite opposite results have been reported according to which no increased susceptibility to infection of the kidneys in hypertensive rats was recorded (*Heptinstall & Stryker* 1962) *Heptinstall* (1962) also showed that hypertension would develop in a certain number of rats in which chronic pyelonephritis was induced either in both kidneys or in the remaining kidney of nephrectomized rats It is possible that the contradictory results of these various experiments are due to differences in the properties of the infecting agent and in the modes of inducing the renal infection

In the present material the mode of inducing renal infection employed by *Heptinstall* (1962) was used and as in his experiments *E coli* was the infecting agent As in his series hypertension did not develop in the presence of a contralateral non infected kidney It did appear however, in a significant number of animals after the removal of the non infected kidney The protective action of this kidney with respect to the development of hypertension in these rats is of an obscure nature The situation bears some resemblance to that seen in cases of renal artery stenosis in dogs in which persistent hypertension develops much more readily if the untouched kidney is removed (*Braun Menendez et al* 1946)

Although there is now conclusive experimental evidence that chronic pyelonephritis may lead to hypertension the mechanisms by which this increase in blood pressure is produced have remained obscure In the classic report on chronic pyelonephritis in man by *Weiss & Parker* (1939) hypertension was ascribed to inflammatory damage and thickening of the walls of the cortical arteries with a consequent alteration in the intrarenal blood flow This explanation seems to have gained wide acceptance although it cannot be valid in all cases since there are cases with severe changes in the vessel walls and no hypertension as well as hypertensive cases without any remarkable vascular changes (*Brod* 1957 *Kleeman et al* 1960 *Lagergren & Ljungqvist* 1962) Moreover *Heptinstall* (1962) stressed that there was usually no diffuse arteriosclerosis in the infected kidney in rats with hypertension due to chronic pyelonephritis This feature was also noted in the present investigation

In the absence of diffuse intrarenal arteriosclerosis in the pyelonephritic kidneys other causes for [illegible] urbed renal blood flow with resulting renal hypertension have to be sought On the basis of findings in man it has been suggested that the altered intrarenal arterial pattern which results from the pyelonephritic damage to the glomeruli would lead to a r [illegible] ical ischaemia and hypertension (*Lagergren & Ljungqvist* 1962) The results of the combined microangiographic and histological examination of the present material have added experimental support to this view Thus the only significant difference between the hypertensive and non hypertensive pyelonephritic rats with respect to kidney histology was the more frequent

occurrence of glomerular lesions in the hypertensive rats. These lesions did not affect the morphological basis for the blood flow through the medulla since the efferent juxtamedullary arterioles which form the vascular supply of the medulla remained intact and in the absence of glomerular tufts appeared as arteriolae rectae verae. In the cortex, on the other hand, glomerular damage resulted in incomplete filling of the arteriole-glomerular units. This difference in reaction is a consequence of the difference in structure between the arteriole-glomerular units in the two regions previously demonstrated in man (*Ljungqvist* 1964). In the juxtamedullary zone the afferent and efferent vessels constitute one continuous vessel, the glomerular arterioles being attached as lateral branches, whereas the glomerular capillaries in the cortex are interconnected between the afferent and efferent vessels.

In the normotensive pyelonephritic rats the change in vascular pattern was less than in the hypertensive rats as a consequence of the less advanced glomerular damage and was presumably incapable of producing a significant degree of cortical ischaemia. It appears possible that hypertension might also have developed in the former group of rats if the rats had remained alive and the glomerular damage progressed.

The pathogenesis of the glomerular lesion is obscure. Identical lesions *in the non-infected areas of pyelonephritic kidneys in hypertensive rats* were described by *Heptinstall* (1962) and were judged to be a consequence of the increased blood pressure (*Heptinstall & Hill* 1967). The occurrence of the glomerular lesion in both normotensive and control rats of the present material indicates that hypertension is not a necessary prerequisite for its development. In the infected but non-pyelonephritic rats and in the control rats the lesion only occurred in the right kidney where the ureter had been temporary occluded. In the pyelonephritic rats the lesion also appeared in the contralateral kidney. It is therefore suggested that the glomerular lesion is a non-specific mode of degeneration induced by various types of pathological situations such as kidney infection, ureteral obstruction, elevated blood pressure etc. If the pathogenetic mechanism is strong or of long duration extensive glomerular degeneration may develop and result *in an* alteration of the intrarenal micro-angioarchitecture. This *alteration* may form a morphological basis for the development of a relative cortical ischaemia and a renal form of hypertension.

There is general agreement that the early phase of renal hypertension is due to a release of renin from the juxtaglomerular cells of the ischaemic cortex. *In hypertension due to renal* artery stenosis this is reflected by hypergranulation of these cells and increase in vasopressor activity of the cortical tissue (*Masson et al* 1964). In this form of hypertension hypergranulation of the juxtaglomerular cells persits also when the hypertensive state is prolonged although plasma *renin* activity declines (*Koletsky et al* 1967) suggesting an altered equilibrium between the

release of renin on one hand and the production and storage of renin in the kidney as reflected by its degree of juxtaglomerular granulation on the other The finding in the present study of a decreased juxta glomerular granulation in pyelonephritic kidneys from normotensive rats suggests that kidney infection in itself is capable of altering the equilibrium between the production and storage of renin on one hand and its release from the kidney on the other The development of hypertension is associated with a further alteration of this equilibrium as judged from the further decrease of juxtaglomerular granulation in the hypertensive rats

SUMMARY

Unilateral chronic pyelonephritis was induced in rats A significant number of these rats developed hypertension provided that the non infected kidney was removed The stereoscopic micro angioarchitecture and histology of the infected kidneys in hypertensive and normotensive rats were compared and JGI determinations were made in all kidneys

The disease induced degenerative changes in the glomeruli These glomerular changes produced alterations in the intrarenal vascular pattern which were more pronounced in hypertensive than in normo tensive animals The altered intrarenal vasculature may be responsible for the development of hypertension since it forms a morphological basis for a relative cortical ischaemia

The JGI values of the infected kidneys were low in both the normo tensive and hypertensive rats

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Acta path microbiol scand 75 583–597 1969

The Department of Physiology Medical School University of Newcastle upon Tyne England the Department of Pathology II University of Umeå the Department of Histology University of Uppsala and the Kristineberg Zoological Station Fiskebackskil Sweden

INVESTIGATION OF GASTRIN ACTIVITY IN PANCREATIC ISLET TISSUE

By

ERIC L BLAIR STURE FALKMER CLAES HELLERSTRÖM HENRIK OSTBERG and DIANA D RICHARDSON

Received 8 x 68

Since the original observation by *Gregory et al* (1960) there have been several recorded instances in which the islet tumour tissue from patients with the Zollinger Ellison syndrome has been found to contain gastrin activity (*Ellison & Wilson* 1964 *Ellison* 1967) The extracts have been shown to possess actions identical with those of extracts of human gastric antral mucosa (*Jackson et al* 1963 *Gregory & Tracy* 1964) *Gregory et al* (1967) provided evidence of the presence in such pancreatic tumours of one or other or both of the gastrin peptides isolated from human antral mucosa (*Gregory et al* 1966 *Bentley et al* 1966)

Although it is generally agreed that the tumour in the Zollinger Ellison syndrome is an insuloma it is still unknown whether any of the various cell types of normal islet tissue can manufacture gastrin Attempts to extract gastrin from normal mammalian pancreatic tissue have not given uniform results (see Discussion) If gastrin producing cells are present in the pancreatic islets they are presumably either A_1 cells or agranular cells In man and common laboratory animals these cells are however only a small proportion of the normal pancreatic tissue (*cf Boquist* 1967) whereas they may be present in large numbers in islet tissue tumours (*Frantz* 19[illegible] *P*[illegible] *et al* 1966 *Cavallero et al* 1967 *Falkmer & Bergdahl* 1967) and in the islet tissue of lower vertebrates (*Falkmer et al* 1964 *Hellerstrom & Asplund* 1966) The A_1 cell

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Request for [illegible] should be sent to Professor Sture *Falkmer* MD Institute of Pathology University [illegible] S–901 87 Umeå 6 Sweden

R[illegible]d in part at the [illegible]n[illegible]ian Congress of Pathology and Microbiology In Copenhagen June 19th–[illegible] 7 (*Falkmer & Bergdahl* 1967)

Th[illegible]k was supported by the *Swedish Medical Research Council* (Projects No K[illegible] X [illegible] 18 0[illegible] K68 12X–718 03 and K68–12X 10[illegible] 4) the *Nordic Insulin Fund* and the U S *Public Health Service* (AM 1 53[illegible])

1 [illegible]pt of a Scholarship for Training in Research Methods from the *Medical Research Council* London

(*Hellman et al* 1962 *Petersson et al* 1962 *Hellerstrom et al* 1964) may be identical with the argyrophil metachromatic cell of *Cavallero & Solcia* (1961) and the D cell of Bloom (*Epple* 1965 *Fujita* 1968) The agranular cell (*Falkmer et al* 1964) is probably identical with the C-cell of *Bowie* (1924)

The present work was undertaken to investigate the possibility that A_1 cells or agranular cells of pancreatic islets produce gastrin Extracts of whole pancreas or isolated pancreatic islet tissue containing significant numbers of A_1- or agranular cells were assayed for their gastrin activity Pancreatic tissue was obtained from a cartilaginous fish the skate *Raya radiata*, and from rattlesnakes (*Crotalus viridis helleri*) (*Hellerstrom & Asplund* 1966 *Östberg et al* 1966) Isolated pancreatic islets composed of about 25 per cent agranular cells were obtained from a marine teleost the daddy sculpin (*Cottus scorpius*) and a cyclostome the hagfish (*Myxine glutinosa*) (*Falkmer* 1961 *Falkmer & Winbladh* 1964) The islet tissue of the daddy sculpin contains A_1 A and B cells in addition to agranular cells whereas that of the hagfish is composed only of B cells and agranular cells

MATERIAL AND METHODS

Tissue Sampling

Whole pancreatic glands were obtained from one adult female skate, 40 cm in length and from 17 adult rattlesnakes of both sexes and 60–150 cm in length The yield of whole pancreatic tissue from the skate was 2 7 g wet weight In the rattlesnakes each pancreas was divided into two parts corresponding to the head and the splenic region because of regional differences in the frequency of A_1 cells in the pancreas (*Hellerström & Asplund* 1966) The total yield of whole snake pancreatic tissue was 2 4 g (head of pancreas) and 3 4 g (splenic region) wet weight The snake specimens were collected in November from animals that had been kept in captivity for about two weeks at a temperature of +25 C Tissue from the skate the sculpin and the hagfish were collected in May and June from animals kept at a water temperature of +8 C

Isolated islet tissue was obtained by removal of acinar parenchyma from the two principal islets of each of 203 adult daddy sculpin of both sexes and with a mean body weight of 122 g In addition pure islet tissues was removed by naked eye *dissection from about 500 hagfish with a mean body length of about 40 cm* and weighing 40–50 g The total amounts of pooled isolated islet tissue obtained from the sculpin and from the hagfish were 1 05 g and 0 42 g wet weight respectively

Immediately after dissection the pancreatic specimens or the isolated islets were weighed on a torsion microbalance and placed in plastic containers immersed in a mixture of 95 per cent ethanol and solid CO_2 at —30 C to —40 C The tissues were thereafter sent in solid CO_2 to Newcastle upon Tyne where they were either extracted for gastrin assay within 24 hours after collection or maintained at —18 C in a deep freeze

Tumour tissue obtained from a patient with the Zollinger Ellison syndrome (*Jackson et al* 1963) was also extracted This tissue had been stored at —18 C for 5 years

Extraction of Tissues

The pancreas and islet tissues were broken up in 4–6 ml of distilled water at 0 C (or in 20 ml HCl pH 1 3 in the case of the rattlesnake tissues) in a loosely fitting Potter–Elvehjem homogenizer rotating at 500 r p m The homogenates were then heated in a boiling water bath for 5 min and the acidified rattlesnake extract only was finally adjusted to pH 7 35 Distilled water extracts were also prepared from

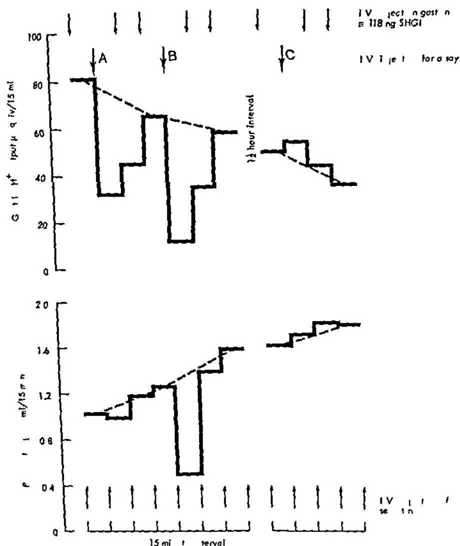


Fig 1

An illustration of the effects (full lines) on the secretion of gastric acid and pancreatic juice by (A) extract of hagfish pancreatic islet tissue (B) extract of sculpin pancreati islet tissue (C) SHG I 12f ng A constant IV d se of secretin was injected at the beginning of each 15 min collection period The dotted lin s represent the pattern of secretion which it is predicted would have occurred had the IV injecti n of the background dose of gastrin continue l uninterrupted every 15 m n and no other IV inject ons been given

the tumour tissue The sup one of th extracts of tun activity All the other exti ac l ne Acet ne prec pitati precipitates were washed v in a sulphuric acid desicca were diss lved in distilled

s of the extracts of rattlesnake pancreas and of ue were immediately assayed for their gastr n rn tants were precipitated with 20 volumes of arried out at +4 C for 24–72 hours The acetone one ether (50 50) and ether an l drie l *in Vacuo* acetone dried powders were water soluble and mediately prior to their assay

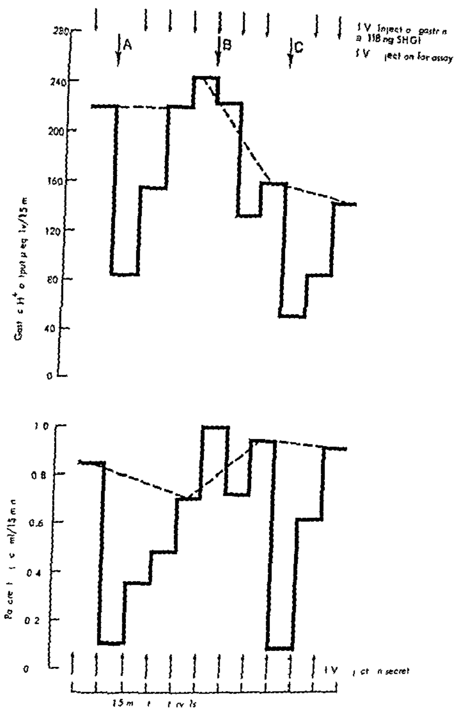


Fig 9

An illustration of the effects (full lines) on the secretion of gastric acid and pancreatic juice by (A) extract of rattlesnake head of pancreas (B) glucagon 500 µg (C) extract of rattlesnake splenic part of pancreas

Assay of Gastrin Activity in the Tissue Extracts

Evidence of gastrin activity in the tissue extracts was sought for by investigating their gastric acid stimulating activity in anaesthetized cats The extracts were injected intravenously and their activities were compared with the gastric acid stimulating activity of a standard gastrin The method of assay was either that of *Blair et al* (1968 a) or a modification of this method (*Blair et al* 1968 b)

In some instances the effect of the extracts on secretin stimulated pancreatic juice was studied simultaneously with their effects on gastric acid secretion The pancreatic duct of the assay animals was cannulated as it enters the duodenum and the volume of pancreatic juice secreted every 15 min was measured There is no basal secretion of pancreatic juice in the anaesthetized cat and a moderate flow of pancreatic juice was maintained by the injection of the same IV dose of secretin extract (*Crick et al* 1950) at the beginning of each 15 min collection period

The earlier work was carried out using the method of bio assay of *Blair et al* (1968 a) which is known to be capable of detecting gastrin equivalent to 50 ng (25 p moles) or more of Synthetic Human Gastrin I (S H G I)[1] (*Blair & Wood* 1968) In applying this method a dose of a standard gastrin extract was given by single rapid intravenous injection at 15 min intervals except on those occasions when an unknown which was to be assayed, was substituted for the standard The gastrin activity of the unknown was determined by comparing the actual rate of gastric acid secretion which according to prediction would have occurred in the same period had there been no substitution of the unknown for the standard This predicted response was assessed by linear interpolation from the acid secretory responses occurring in the 15 min periods on either side of the assay half hour (Figs 1 2 and 3) The percentage relationship between these actual and predicted secretory rates was referred to as the response The dose of gastrin which produced such a response was determined from a dose response curve and the results were expressed in ng of S H G I

In the modification used later on in our investigation the gastrin activity of the unknown was determined by comparing the actual rate of acid secretion in the 15 min following its injection with the acid secretion which according to prediction would have occurred in the same period had there been no substitution of the unknown for the standard gastrin The predicted response was again assessed by linear interpolation from the acid secretory responses occurring in the 15 min periods on either side of the assay 15 min (Fig 4) The percentage relationship between the actual and predicted secretory responses was referred to as the response to the unknown In calculating the dose of gastrin to which this response was equivalent it was considered in relation to an adjacent paired similarly calculated response to a known dose of gastrin

RESULTS

The effects on the secretion of gastric acid and pancreatic juice of the extracts of islet tissue from the hagfish (115 mg) and the sculpin (48 mg) are illustrated in Fig 1 The effects on gastric acid secretion were not significantly different from the results which would be expected from the injection of saline alone (Fig 5) and it is concluded that any gastrin activity in the extracts must have been less than that which is equivalent to 50 ng S H G I The effect of the injection of 126 ng S H G I in the same assay animal is also illustrated in Fig 1 While the injection of the hagfish extract produced only a very small reduction in the rate of flow of secretin stimulated pancreatic juice the sculpin extract caused an appreciable and highly significant reduction

The effects of the injection of the extracts of rattlesnake pancreas on the responses of the assay animal are illustrated in Fig 2 There

[1] Prepared by Imperial Chemical Industries Ltd England

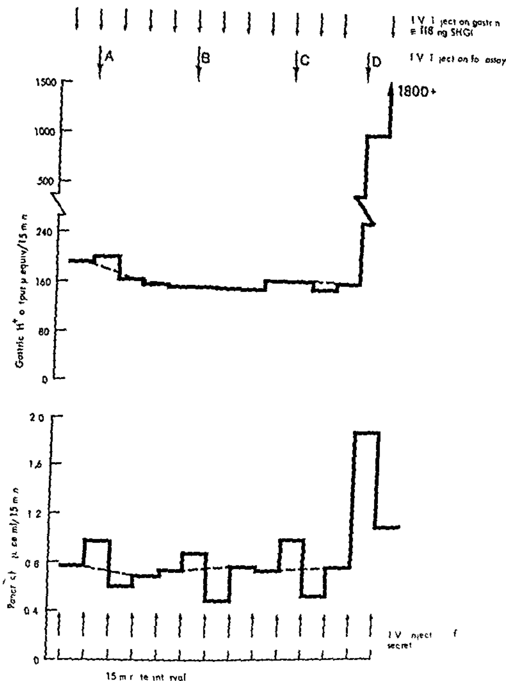


Fig 3

An illustration of the effects (full lines) on the secretion of gastric a id and pau creatic juice by (A) glucagon 400 μg (B) glucagon 600 μg (C) glucagon 400 μg (D) extract of 397 mg of tumour tissue from a patient with the Zollinger-Ellison syndrome Further details are given in Fig 1

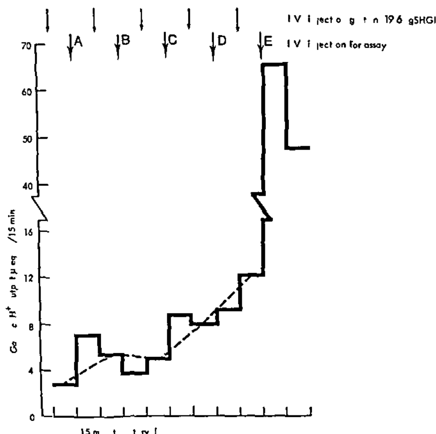


Fig 4

An illustration of the effects (full lines) on the secretion of gastric acid by (A) Gastrin ?75 ng S H G I (B) extract of skate pancreas (64 mg) (C) Gastrin 275 ng S H G I (D) Gastrin 14 ng S H G I (E) extract of 82 μg of tumour tissue from a patient with the Zollinger-Ellison syndrome The dotted lines represent the pattern of secretion which it is predicted would have occurred had the I V injection of the background dose of gastrin continued uninterrupted every 15 min and no other I V injections had been given

was no evidence for a stimula ry effect on gastric acid secretion by extracts of either the head or plenic regions of the pancreas In both instances however there was p efound prolonged inhibition of secre tin stimulated pancreatic secretion the effect was more prolonged following the injection of the extract of the head of pancreas than of the splenic part of the pancreas The effect of a rapid intravenous in jection of ox glucagon in the same animal is also illustrated in Fig 2

The effect of glucagon c the flow of secretin stimulated pancreatic juice was typical of that frequently found with such doses in these experimental animals there was an increase in the expected rate of

[1] Crystalline gluc gon as the hydrochloride Eli Lilly & Co U S A

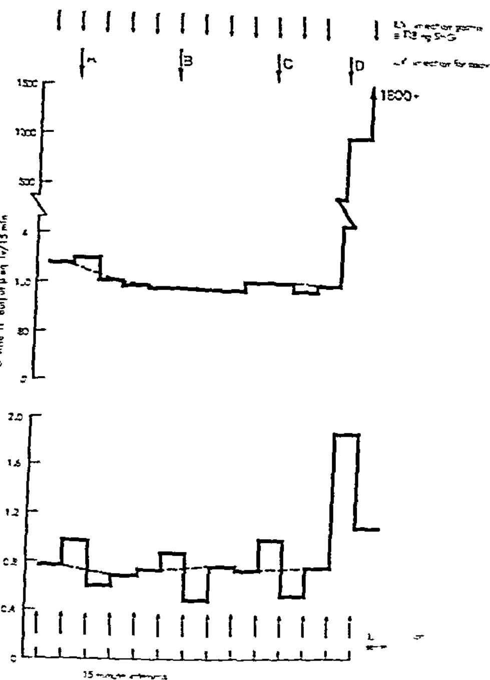


Fig 3.

...n illustrati n o the effects full lines on th. secreti n of gastric a d an pancreatic juice by (A glucagon. 3 0 µg. B glucag n, 6 µg. (C) glucag n, 6 µg ...) extract of 3 mg of tum ur ti sue from a patient with the Zo nger-Ellis n syndrome. Further deta s are given in Fig. 1

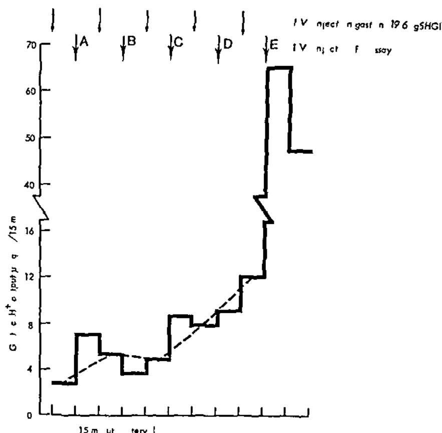


Fig 4

An illustration of the effects (full lines) on the secretion of gastric aci l by (A) Gastrin 27 5 ng S H G I (B) extract of skate pancreas (64 mg) (C) Gastrin 27 5 ng S H G I (D) Gastrin 14 ng S H G I (E) extract of 82 μg of tumour tissue from a patient with the Zollinger-Ellison syndrome The dotted lines represent the pattern of secr tion which it is predicted would ha e occurred had the I V injection of the background dose of gastrin continued uninterrupted every 1 min and no other I V inject ons had been given

was no evidence for a stimulatory effect on gastric acid secretion by extracts of either the head or splenic regions of the pancreas In both instances however there was a profound prolonged inhibition of secretin stimulated pancreatic secretion the effect was more prolonged following the injection of the extract of the head of pancreas than the splenic part of the pancreas The effect of a rapid intravenous injection of ox glucagon[1] in the same animal is also illustrated in Fig

The effect of glucagon on the flow of secretin stimulated pancreatic juice was typical of that frequently found with such doses in these experimental animals there was an increase in the expected rate

[1] Cry talline glucagon a the h drochloride Eli Lilly & Co I A

secretion in the 15 min period immediately following the injection of glucagon followed by a reduction in the second 15 min period. There was however no significant reduction in the total secretion of pancreatic juice in the 30 min following the injection of glucagon compared with the total secretion which was expected to occur in that same period if glucagon had not been injected. The effect of glucagon on the flow of secretin stimulated pancreatic juice is also illustrated by the results from another experimental animal (Fig 3). There was clearly no appreciable effect of glucagon on gastrin stimulated acid secretion in this experiment. The effect of the rapid intravenous injection of glucagon on the gastric acid secretory responses to the standard gastrin extract was studied on 9 occasions in 4 animals and the results are shown in Table 1.

TABLE 1

The Effect of the Simultaneous Rapid I V Injection of Glucagon on Gastric Acid Secretory Responses to Gastrin

Dose of glucagon (μg)	Dose of gastrin injected every 15 min expressed as ng S H G I	Gastric acid secretory response in assay ½ hr expressed as per cent of predicted response in absence of glucagon injection
50	392	98 5
75	392	78 0
100	392	124 5
350	392	112 0
350	118	111 0
450	118	98 0
500	118	88 5
500	392	113 5
600	118	101 5
		Mean 102 8 S D ± 13 3

The gastrin dose is injected at 15 min intervals. The acid secretory response occurring in the 30 min following the injection of glucagon is compared with the response it is expected would have occurred if no glucagon had been injected (See also Figs 2 and 3)

There was a large stimulatory effect on both gastric acid secretion and the flow of pancreatic juice following the intravenous injection of an aqueous extract of 327 mg of the stored tumour tissue from the patient with the Zollinger Ellison syndrome (Fig 3).

The acetone extract of skate pancreas (64 mg) which was assayed for gastrin activity by the modification of the method of Blair *et al* (1968 a), a more sensitive and more precise method of assay, was estimated to have a gastric acid stimulating activity equivalent to 16 6 ng (8 3 p moles) S H G I (Fig 4). The marked stimulation of gastric acid secretion in the same experiment by the acetone extract from 82 μg of the tumour tissue from the patient with the Zollinger Ellison syndrome is also illustrated in Fig 4 (the pancreatic duct was not cannulated in the animal used for these studies and no secretin was injected).

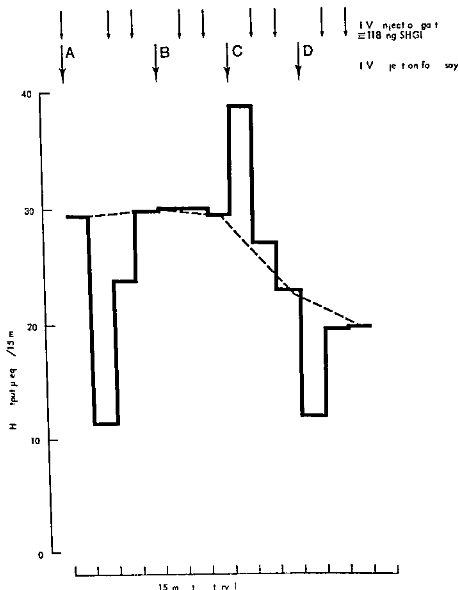


Fig 5

An illustration of the effects on the secretion of gastric acid (full lines) of the injection of (A) saline (B) the same dose (C) a larger dose and (D) a smaller dose of gastrin than the background level of gastrin. Further details are given in Fig 4

DISCUSSION

Attempts to extract gastrin-like activity from the normal mammalian pancreas have so far yielded conflicting results. With the exception of the studies of *Konaka et al* (1965, 1966) no active principle has been

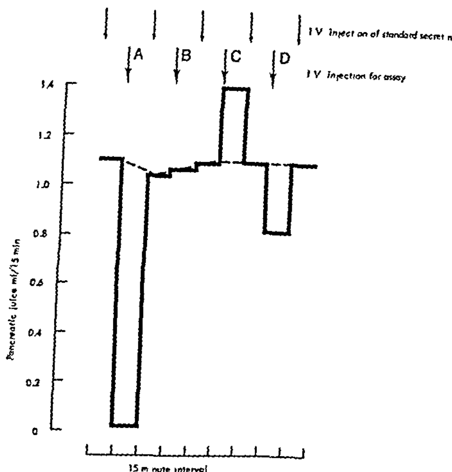


Fig 6

An illustration of the effects on the secretion of pancreatic juice (full lines on the injection of (A) saline (B) the same dose (C) a larger dose and (D) a smaller dose of secretin than the background standard dose of secretin The dotted line represents the pattern of secretion which it is predicted would have occurred had the I V injection of the background dose of secretin continued uninterrupted every 15 min and no other I V injections had been given

detected these workers reported a stimulation of gastric acid secretion with an extract prepared from hog pancreas Gastrin is however rapidly destroyed by enzymes present in the pancreatic acinar cells and studies have therefore been performed also with atrophic pancreas From chronically damaged non tumour containing pancreas in man *Zollinger et al* (1962) and *Elliot et al* (1964) extracted a gastric secretagogue and *Zollinger et al* (1962) detected a similar activity in extracts of pooled dog pancreas previously made atrophic by ligation of the pancreatic ducts By contrast neither *McIlrath et al* (1963) nor *Osborne et al* (1963) succeeded in demonstrating a gastric secretagogue in atrophic dog pancreas

In our study every precaution was taken to minimize enzymic de

struction of gastrin during sampling transport and extraction of the whole pancreas and pancreatic islet tissue The specimens were rapidly frozen after their collection and were maintained in the deep frozen state before their extraction in the cold Immediately after the extraction process all the extracts were boiled The danger of enzymic destruction of gastrin in the whole pancreas was particularly great and for this reason the rattlesnake pancreas was extracted at acid pH Despite these precautions none of the extracts showed gastrin activity equivalent to 50 ng (25 p moles) or more of S H G I By contrast an extract of metastatic tumour tissue which had been stored for 5 years in the cold and was obtained from a patient with the Zollinger Ellison syndrome possessed considerable gastrin activity *Osborne et al* (1963) also were unable to detect gastrin activity in pancreatic islet nodules of the angler fish (*Lophius piscatorius*)

It is possible that gastrin activity was present in these extracts of pancreas and pancreatic islet tissue but that its effect on gastric acid secretion was counteracted by the presence of other biologically active agents which have an inhibitory effect on gastrin stimulated acid secretion The extracts were not specifically tested for any such inhibitory effect If however an inhibitor is present the time course of its action must be the same as that of gastrin because the pattern of the gastric acid secretory responses to the background doses of gastrin following the injection of extracts from the sculpin hagfish and rattlesnake (Figs 1 and 2) do not differ from those expected when saline alone was injected in place of gastrin (Fig 5) The rate of gastric acid secretion to the background dose of gastrin did decline as the experiments proceeded but this is a usual characteristic of responses to the repeated injection of the standard gastrin alone in these assay animals (*Blair* 1967) as is illustrated in Fig 5 Nevertheless the acetone dried extract of sculpin pancreatic islet tissue (Fig 1) and the acid aqueous extracts of rattlesnake pancreas (Fig 2) did produce significant inhibition of secretin stimulated pancreatic secretion The relatively prolonged inhibitory effect on secretin stimulated pancreatic secretion is emphasized by the shortlived reduction in flow of pancreatic juice which resulted when an I V injection of saline was substituted for secretin (Fig 6) There is some experimental evidence indicating that the secretin stimulated secretion of pancreatic juice may be depressed by prostaglandins (*Hutt* 1968) Perfusion of the mucosal surface of the rat stomach with Prostaglandin E_1 is known to inhibit the secretion of pentagastrin stimulated acid secretion by 30–80 per cent (*Ramwell & Shaw* 1968) and *Robert et al* (1967) have described the inhibition of gastric acid secretion in the dog by certain of the prostaglandins given intravenously

The fact that the extract from the head region of snake pancreas had a more prolonged inhibitory effect on the secretin stimulated pancreatic secretion than had the extract of the splenic region could be explained if the inhibiting factor occurs in the acinar parenchyma since the main

bulk of snake islet parenchyma occurs in the splenic region (*Hellerstrom & Asplund* 1966) If this is so then it might also explain why the extracts of sculpin principal islets where some acinar parenchyma may remain peripherally after rapid naked eye dissection inhibited the secretin stimulated pancreatic juice secretion more than those of hagfish islet parenchyma where no acinar tissue occurs close to the islet organ (*Falkmer & Winbladh* 1964)

Previously published reports on the cellular composition and the glucagon content (*Falkmer* 1966) of the tissues used in the work suggest that the extracts might contain glucagon There is however conflicting evidence about the effect of glucagon on gastric acid secretion and no well substantiated evidence that it will inhibit gastrin stimulated acid secretion Furthermore the fact that extract of hagfish islet tissue did not stimulate acid secretion cannot be explained by the simultaneous presence of glucagon in the extract because this tissue contains only *B* and agranular cells and it is most unlikely that quantities as great as 350 600 μg glucagon were present in the other extracts and yet these quantities of crystalline glucagon did not have any appreciable inhibitory effect on gastrin stimulated acid secretion (the apparent small inhibitory action illustrated in Fig 2 was unusual and is not a consistent effect—Table 1 and Fig 3) The effects of glucagon on secretin stimulated pancreatic juice (Figs 2 and 3) also do not suggest that these quantities of glucagon were present in the extracts

There is a biphasic effect by glucagon on the output of secretin stimulated pancreatic juice with an apparent increase in flow during the first 15 min after its injection and a decrease in flow in the second 15 min with no appreciable alteration in net flow over the 30 min period *Necheles* (1957) also described a biphasic effect of glucagon on the flow of pancreatic juice in the dog although the phase of depressed secretion was considerably more prolonged This phenomenon could be the outcome of vasomotor changes in the pancreas or could result from contraction of the smooth muscle of the pancreatic ducts by glucagon during the first 15 min after its injection Earlier reports of the action of glucagon on gastrointestinal muscle are however confined to the description of an inhibitory effect on gastric and colon motility (*Stunkard et al* 1955 *Sporn & Necheles* 1956)

The results obtained with sculpin and hagfish islet tissue do not provide any evidence to support the view that the agranular cells of pancreatic islet parenchyma produce gastrin This is not surprising if the working hypothesis of Falkmer and his colleagues (*Falkmer et al* 1964 *Falkmer & Winbladh* 1964 *Falkmer & Bergdahl* 1967) is true These workers have suggested that these cells are immature precursor cells—notably to the B cells—without any clear ultrastructural or functional signs of active secretion of their own

The single investigation using a more sensitive and precise method of assay revealed gastric acid stimulating activity equivalent to 16 6 ng

(8 3 p moles) S H G I (Fig 4) in the acetone extract (64 mg) from 27 g (wet weight) of skate pancreas It is clearly necessary to repeat the experiment on the skate pancreas and to reinvestigate the other pancreatic and islet tissues with the more sensitive method of assay of gastric acid stimulating activity *If it is confirmed that gastrin is present* in extracts of the skate pancreas it must be in extremely small quantities compared with those which can be extracted from human islet cell tumour tissue (Fig 4) This is perhaps not surprising if the presence of gastrin is restricted to A_1 cells which constitute only a few per cent of the endocrine tissue of the skate pancreas (and consequently an even smaller fraction of the total mass of the compact gland) On the other hand if cells of similar structure are responsible for producing the gastrin of the pyloric antrum (*Solcia et al* 1967 *Coalson* 1968) *these also are a very small proportion of the total number of* cells in pyloric antral mucosa and yet almost 400 times the amount of gastrin activity has been extracted from the same weight of human pyloric antral mucosa (*Blair* 1964) as was extracted from the skate pancreas in this instance Nevertheless if it is confirmed that gastrin activity however small is present in normal pancreatic tissue especially in association with A_1 cells this will help to explain how it is that cells resembling these pancreatic islet cells can produce gastrin in large quantities when they undergo neoplastic change

SUMMARY

1 Attempts were made to extract gastrin activity from the pancreas and pancreatic islet tissue of a number of species by a method known to be successful in the extraction of gastrin activity from comparable weights of gastric antral mucosa in mammals and of insuloma tissue in patients with the Zollinger Ellison syndrome Whole pancreatic tissue *containing A_1 cells was obtained from skate and snake Isolated* islet tissue containing agranular cells was obtained from sculpin and hagfish

2 Evidence of gastrin activity in the extracts was sought by assay of their gastric acid stimulating activity in the anaesthetized cat No such activity was found by a method which would be expected to detect 50 ng or more of Synthetic Human Gastrin I

3 An extract of skate pancreas was on a single occasion tested with a more sensitive method for detecting gastrin activity Gastric acid stimulating activity equivalent *to 16 6 ng (260 ng/g wet tissue weight) Syn*thetic Human Gastrin I was found This small quantity of gastrin like activity is contrasted with that obtained from gastric antral mucosa and from tumour tissue in the Zollinger Ellison syndrome

4 The results are in accord with the hypothesis that the agranular cells of pancreatic islet tissue do not secrete any hormone

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The Bacteriological Laboratory Ullevål Hospital Oslo Norway

STUDIES ON THE COMPLEMENT FIXATION TEST WITH *MYCOPLASMA PNEUMONIAE* ANTIGEN

3 *Observations on the Development of Complement Fixing Antigen in Broth Culture*

By

JAN LIND

Received 30 viii 68

Mycoplasma pneumoniae antigen for diagnostic complement fixation tests (CFT) is usually produced by cultivating the organism in the broth medium developed by *Chanock et al* (3) centrifuging down the organisms after one week s incubation or more and use of the deposit after treatment to reduce its anticomplementary properties (1 3 7 15) Several recent studies have contributed to a further characteriza tion of the antigen Its solubility in organic solvents was described by *Kenny & Grayston* (15) *Prescott et al* (23) and *Sobeslavsky et al* (25) demonstrated that the CF antigen of *M pneumoniae* is a lipid *Prescott et al* (22) characterized the antigen as a phospholipid hapten *Sobeslav sky et al* (25 26) found that the antigen is probably a constituent of the outer cell membrane of *M pneumoniae*

The present investigation deals with the growth pattern of *M pneu moniae* in fluid medium with special reference to the development of centrifugable and non centrifuged CF antigen in broth culture corre lated with the development of colony forming units (CFU) The effect of sonication on the CF antigen was investigated

MATERIAL AND METHODS

The Bård strain of *M pneumoniae* (7) was cultivated in normal PPLO broth and on normal PPLO agar as described by *Chanock et al* (3 4) but with some minor modi fications as referred to in (7) Serum from a rabbit immunized with the Bård strain cultivated on a medium made up of rabbit meat extract and normal rabbit serum (7) was used as a positive serum for titration of the antigenic preparations The serum had a CF titre of 1 640 when tested against concentrated antigen (cf Table 3) and was used in the dilution 1 20 throughout the present study —*The CFT technique* was as described by *Bradstreet & Taylor* (2) and referred to in (7) in more detail

Counting of CFU in broth culture was done by plating on to PPLO agar placing a layer of blood agar on the agar plate with colonies and counting of the zones of haemolysis The principle of this technique is the same as that used by *Freimer et al* (8) to demonstrate production of haemolysin in L forms and protoplasts of Group A Streptococci *Freundt* (9) reported that *M pneumoniae* possesses haemolytic

properties *Clyde* (6) and *Somerson et al* (29) described simultaneously that the placing of a blood agar layer on agar plates with colonies of *M pneumoniae* resulted in the formation of zones of haemolysis around the colonies In the present investigation the broth cultures were titraded in sterile normal PPLO broth in $\log_{10}$ dilution steps 0 05 ml of the dilutions was spread carefully on normal PPLO agar in plastic Petri dishes with a diameter of 9 cm The plates were sealed airtight when free fluid was no longer visible on the agar surface and incubated at 35 C After incubation for 8 days 2 ml of 10 per cent washed sheep red cells was mixed evenly with 5 ml of PPLO agar medium Difco at 50 C and the mixture was immediately poured on each plate which was placed in a strictly horizontal position The counting of the haemolytic plaques was done after 3 days incubation with the blood agar layer either macroscopically or by means of a hand lens

The broth cultures were centrifuged in a Sorvall superspeed RC-2B cooling centrifuge Optical density was measured in a Hilger Biochem Absorptiometer Sonication was performed in a MSF Ultrasonic Power Unit model 60 w using a probe with a diameter of 2 5 mm making possible sonication of volumes as small as 0 4 ml

EXPERIMENTAL

Broth culture of *M pneumoniae* Bård strain which had been kept frozen at —50 C for 2 years and 4 months was thawed and inoculated on normal PPLO agar After 1 week s incubation at 35 C numerous colonies of varying sizes appeared The strain was subcultured 3 times on agar medium at intervals of 3–5 days the last sub culture showed confluent growth (Fig 1) and this was used for inoculation of normal PPLO broth After incubation for 4 days the broth culture was filtered throughs several layers of sterile gauze to remove the agar masses and then transferred to 11 flasks containing normal PPLO broth Each flask contained 200 ml of normal PPLO broth and was inoculated with 20 ml of filtered broth culture The cultures were incubated at 3[illegible] C Floating colonies (spherules" (15)) were just visible macroscopically from day 4–5 The cultures were examined after incubation for from 0 to 10 days The following examinations were performed in all the cultures

1) The CF titre and anticomplementary (AC) titre were measured in non centrifuged broth culture

a) after inacti[illegible]ation at 5[illegible] C for 30 min

b) after inactivation at 56 C for 30 min followed by sonication for 20 min

2) The broth culture was centrifuged at 23 000 C for 45 min The upper part of the supernatant was carefully withdrawn (SN 1) The deposit was resuspended in 1/100 vol diluent (DEP 1) The CF titre and the AC-titre were measured in SN 1 after inactivation at 56 C for 30 min The CF titre and the AC-titre were measured in DEP 1

a) after inactivation at 56 C for 30 min

b) after inactivation at 56 C for 30 min followed by sonication for 20 min

c) after boiling in a water bath for 35 min

3) The supernatant SN 1 was centr fuged at 33 000 G for 90 min The upper part of the supernatant was carefully w thd awn ([illegible] 2) 1/100 vol diluent was added to the deposit (DEP 2) The CF tit[illegible] nd the AC tit e were measured in SN 2 after inactivation at 56 C for 30 m n T[illegible] F t[illegible] nd the AC titre were measured in DEP 2 after inactivation at 5[illegible] [illegible] 20 min The DEP 2 could not be resuspended unsonicated

4) The number of CFU wa[illegible] c[illegible]nted in the br[illegible]th culture

5) Optical den[illegible]ity was meas[illegible]red in unt[illegible] th culture and in DEP 1 after inactivation at 56 [illegible] f[illegible]r 30 min

6) pH was meas[illegible]r d [illegible] the broth [illegible]ultu e

All the titrations f[illegible]r [illegible] F and AC t[illegible]res were made [illegible] dilution steps The AC titres of th[illegible] tions were m[illegible]ed by replac[illegible]ng [illegible] siti[illegible]e serum by diluent (Ca Mg buffered d[illegible]ent w th pH 7 2 21) The measu ing of CF titre in non-centrifuged broth cultu[illegible] d in supernata[illegible] will be described in more detail under Results and Discu[illegible] The son[illegible] t[illegible] time (20 min) was determined after pr liminary experiments [illegible] harvested from broth culture after 10 day[illegible] incul ation was sonicated f[illegible] 10 d ff[illegible]nt periods of time from 30 sec up to 55 min A gradual increase in C[illegible] titre w[illegible] h[illegible] ed after [illegible]onication for up to

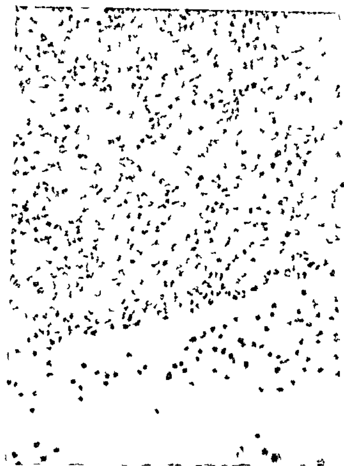


Fig 1

Confluent growth of *M pneumoniae* strain Bård on agar medium inoculated with an agar culture by the agar block technique Note single colonies peripherally Magnification × 100

20 min Sonication for 20–55 min did not result in any further change of titre During sonication the preparations were kept in an ice bath and the temperature never exceeded 25 C

RESULTS

Fig 2 shows that the number of CFU/ml broth culture increased × 30 during the first 3 days of incubation (The numbers were estimated from the platings of the 10^{-4} dilutions of the broth cultures except on day 8 when it was calculated from the 10^{-3} dil) From day 6 on the number of CFU/ml decreased very rapidly The pH dropped from 7 6 on day 0 to 5 7 on day 7 control broth medium showed pH 7 6–7 5 throughout the period of incubation

Fig 3 shows the development of the CF and AC titres in DEP 1 The *CF titres* of deposits from broth cultures in the log phase was un

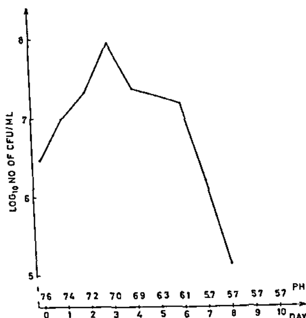


Fig 2

The development of the number of colony forming units in broth culture of *M pneumoniae* strain Bård The pH values of the broth cultures are also given

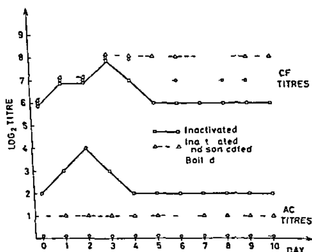


Fig 3

The development of the complement f xation (CF) titres and the anticomplementary (AC) titres of the deposits from broth culture of *M pneumoniae* strain Bård after centrifugation at 23 000 C for 45 min and resuspension of the deposits in 1/100 vol diluent The deposits were tested a) inactivated at 56 C for 30 min b) inactivated and then sonicated for 20 min c) boiled in a water bath for 30 min

tively in 1/100 vol diluent Sonication for 20 min yielded homogenous preparations which showed positive CFT with titres from 1 4 to 1 32 with no regular development during the incubation period The ratio antigen amount in DEP 2/antigen amount in SN 1 varied from 1 25 to 1 300 After centrifugation of the broth cultures at 23 000 G for 45 min still more centrifugable antigen was thus present in the supernatant but the relative yield in the deposits after a renewed centrifugation of the supernatant at 33 000 G for 90 min was significantly less than the gain following the first centrifugation

SN 2 showed positive CFT of exactly the same strength as SN 1 *i e* the titre curve for SN 2 is identical with that for the non centrifuged broth cultures (Fig 4)

Fig 5 shows the development of optical density in non centrifuged broth culture undiluted and in the DEP 1 diluted 1 16 (DEP 1 from day 4 and 5 were combined and also DEP 1 from day 6 and 7 in order to obtain volumes large enough for this examination) The measurements were performed in a Hilger Biochem Absorptiometer in standard 16 mm tubes and with a filter with peak transmission at wave length 545 mμ The optical density of the non centrifuged broth cultures changed very little throughout the incubation period In contrast to this the deposits showed a marked continous increase of density for as long as up to day 9

DISCUSSION

A characteristic feature in the growth of *M pneumoniae* in broth culture is the formation of clusters containing several elementary bodies *Kenny & Grayston* (15) noted that spherules (fluid medium colonies) appeared in broth cultures of *M pneumoniae* In the present study they were evident from day 4-5 *Kim et al* (16) observed in the electron microscope that *M pneumoniae* grown in broth culture was characterized by the presence of spherical elementary bodies (250-300 mμ) appearing in clusters *Furness et al* (10 11) presented experimental evidence indicating that broth culture of *M pneumoniae* contains both clusters and single elementary bodies they concluded that *M pneumoniae* replicate by binary fission and that the aggregates thus formed separate into single elementary bodies with difficulty —It seems reasonable to assume that a wide variation in particle size exists in broth cultures of *M pneumoniae* *Clark* (5) demonstrated a wide variation in particle size in two other mycoplasma species namely *M hominis* and *M gallisepticum* in broth culture *Lynn* (21) discussed the possibility that mycoplasma particles of different size may have different immunological properties

The wide variation in particle size which probably characterizes the growth of *M pneumoniae* in broth culture manifests itself both in the great variability of colony size following the inoculation of broth culture

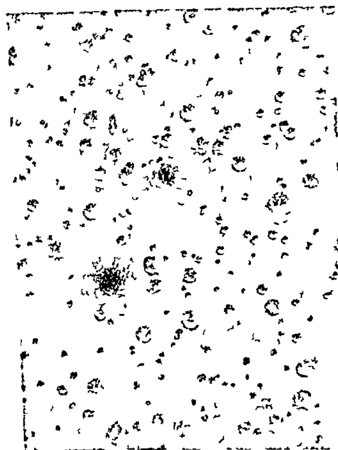


Fig 6

Colonies of *M pneumoniae* strain B rd on agar medium inoculated with a broth culture Note marl ed variation in colony size Magnification × 100

on agar medium (Fig 6) and in the corresponding variability in the dia meter of the zones of haemolysis after this type of agar culture has been overlaid with a blood agar layer (Fig 7) It also explains why CF antigen may be harvested from broth culture by centrifugation at very different velocities as noted by *Chanock et al* (3) and *Jansson* (13) and also demonstrated in the present investigation

Aggregates of elementary bodies can a priori be formed by 1) an in hibition of the separation of the daughter cells following binary fission as suggested by *Furness et al* (10 11) or 2) by an agglutination of growth elements The fall in the CF titres of the inactivated deposit antigens which in the present investigation occurred from day 3 (Fig 3) may be explained by an agglutination of growth elements lead ing to a reduction of the total antigen surface Both mechanisms men tioned above for the formation of aggregates of elementary bodies may be present in broth cultures of *M pneumoniae*

indirect haemagglutination test. The present investigation yields information on the effect of sonication on *M. pneumoniae* CF antigen. The relevance of these results to the growth dynamics of *M. pneumoniae* in broth culture has been discussed above. It is also evident that the CF antigen of *M. pneumoniae* is highly resistant to sonication. Sonication of diagnostic CF deposit antigens of *M. pneumoniae* results in 1) an increase of the CF titre of "late" deposit antigens and 2) a reduction of the AC titre. In the present investigation these two effects together gave an increase of the distance between CF and AC titre from 4 to 7 $\log_2$ dil. steps in the late deposit antigens. 3) Sonication results in a pronounced homogenization and stabilization of the deposits. The sensitivity, specificity and stability of antigens treated with a combination of boiling and sonication deserves to be studied.

The present cultivation experiments of *M. pneumoniae* in broth were generally characterized by an early culmination of both number of CFU in broth culture and CF titres in the deposits and by a small difference between start and maximum titre in CFT. Both these features reflect the use of heavy inoculates which the present author has found important in the propagation of *M. pneumoniae* in broth culture for production of high titred CF antigens (7). *Low & Eaton* (20) found that the highest inoculates resulted in the earliest and highest maximum number of CFU, whereas the smallest inoculates gave greatest difference between start and maximum number of CFU. The number of CFU increased in the present investigation more than the CF titre; this was also found by *Somerson et al.* (27); the explanation may be the presence of "dead" antigen in the inoculates. The pH drop in the broth cultures (Fig. 2) reflects the ability of *M. pneumoniae* to produce acid in a medium containing glucose.

The AC properties of deposit antigens of *M. pneumoniae* have been treated by several methods referred to and discussed in (7). In addition the present investigation yields information on the effect of sonication (Fig. 3).

Fig. 5 shows that only very small changes in *optical density* took place in non-centrifuged broth culture during the growth of *M. pneumoniae*. Broth cultures incubated for 5 days or more had only a slightly higher density than those incubated for 0–4 days. This finding is in agreement with *Smith's* (24) general statement of the low sensitivity of the turbidimetric method in quantitation of growth of mycoplasmas in fluid media. However, the optical density of the deposits increased markedly and continously up to day 9 when it culminated. The CF titres of the deposits culminated on day 3 (Fig. 3). These findings indicate that the deposits are partly made up by components of the growth medium and that the content of medium constituents in the deposit antigens increases throughout the incubation period (up to day 9). Growth medium constituents in deposit antigens of *M. pneumoniae* have been discussed in connection with the AC effect of the anti-

gens (27) and in connection with the occurrence of low titred cross reactions in the CFT between *M pneumoniae* and other human myco plasma species (14 17 18)

SUMMARY

The development of complement fixing antigen in broth culture of *M pneumoniae* was correlated with the development of colony form ing units and the effect of sonication on the antigens from different growth phases was investigated Sonication of the antigens harvested from the broth cultures in the involution phase resulted in a significant increase in CF titre whereas sonication did not influence the CF titre of log phase antigens in these experiments Sonication reduced the anti complementary effect of antigens from all growth phases and gave pronounced homogenization and stabilization of the antigens The findings are discussed in relation to the growth dynamics of *M pneu moniae* in broth culture and their practical importance in the produc tion of diagnostic antigens is discussed

The CF titre in non centrifuged broth culture and in the supernatant after centrifugation was measured by direct CFT titration of the pre parations after inactivation at 56 C for 30 min The amount of CF antigen in non centrifuged broth culture measured by this method was 5–6 × that in the deposits after centrifugation at 23 000 G for 45 min Further centrifugation of the supernatants at 33 000 G for 90 min gave only a minimal change in the ratio centrifuged/total antigen The nature of the non centrifuged CF antigen of *M pneumoniae* in broth culture is discussed

By correlation of turbidimetric and serologic data evidence was found for the occurence of increasing contamination of *M pneumoniae* deposit antigens with broth medium constituents on prolonged incuba tion

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Ampicillin Alpha aminobenzylpenicillin sodium (Astra)

The dilutions of the antibiotics were based on the weight of the substances The weight of the active part of penicillin G is 93 9 per cent of its total weight The corresponding values for penicillin V and ampicillin are 90 0 and 94 1 per cent These differences were ignored The figures given thus denote the activity expressed in units of weight of the respective penicillin salts Plates containing antibiotics were stored at +4 C and used within 24 hours after preparation

Bacteria

H influenzae 80 strains isolated from discharge from the ears of children with acute otitis media *H parainfluenzae* 25 strains isolated from the throat of healthy adults The strains were maintained by subculture every third day on haematin agar plates which were incubated at +37 C in air The strains were maintained at least 3 weeks after isolation before they were tested

Identification of Haemophilus influenzae and Haemophilus parainfluenzae

Both species Gram negative coccoid rods or rods in filaments Stimulation of growth around the inoculum with staphylococci on blood agar plates (symbiosis plates) No haemolysis

Growth on meat extract agar

	without X or V factor	with X factor	with V factor	with X and V factor
H influenzae		–	–	+
H parainfluenzae			+	+

Determination of the Minimum Inhibitory Concentration (MIC) and 50 per cent Inhibitory Concentration (IC_{50})

Bacteria from colonies on haematin agar plates incubated for 18–20 hours were suspended in broth and inoculated with a multi point inoculator (2) into haematin agar plates and haematin agar plates containing two fold dilutions of the antibiotics The volume of the inoculum was about 0 02 ml The number of colony producing bacteria per inoculum was 10^5–10^6 Two series of plates were inoculated. One of the series was incubated in air at +37 C the other in 8–10 per cent CO_2 atmosphere at 37 C in closed containers with the same relative humidity Each set of strains examined included 2 reference strains Incubation time 18 hours

Dense even confluent growth about 1 cm in diameter appeared on haematin agar plates without antibiotic

Readings of Results

By comparison with the growth on the plates without antibiotics the results were graded as follows 4 dense even confluent growth 3 confluent growth but less dense than 4 2 growth of small but readily recognized colonies 1 growth of small barely visible colonies 0 no visible growth With the use of this grading system a symmetric distribution of the growth of the inocula was obtained The results were read with the naked eye

The 50 per cent inhibitory concentration (IC_{50}) was said to be that concentration of antibiotic which gave growth degree 2 according to the Karber method (Finney 1947 *Reyn et al* 1963) the minimum inhibitory concentration (MIC) that concentration which gave no visible growth

RESULTS

Haemophilus Influenzae

The lowest concentrations (MIC) of ampicillin penicillin G and penicillin V that inhibited all strains incubated in air were 0 6 2 4 and 9 6 µg/ml respectively The concentration of penicillin G and V neces

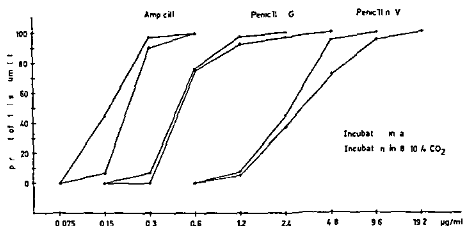


Fig 1
Susceptibility of 80 *Haemophilus influenzae* strains to penicillin G penicillin V and ampicillin Minimum inhibitory concentration µg per ml

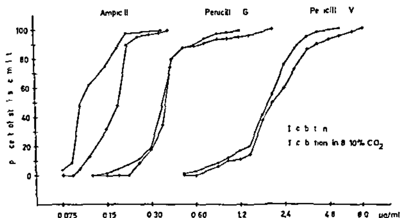


Fig 2
Susceptibility of 80 *Haemophilus influenzae* strains to penicillin G penicillin V and ampicillin 50 per cent inhibitory concentration µg per ml

sary to inhibit all strains on incubation in CO atmosphere were twice as high while no difference in this respect was found for ampicillin

The distribution of the strains according to susceptibility to ampicillin and to penicillin V differed widely with the type of ambient gas The corresponding variation in susceptibility to penicillin G was only slight Thus 45 per cent of the strains were inhibited by 0 15 µg ampicillin/ml on incubation of the strains in air against only about [illegible] per cent of the strains when incubated in CO_2 atmosphere 4.8 µg penicillin V/ml inhibited 95 per cent of the strains incubated in air compared with about 70 per cent incubated in CO_2 atmosphere (Fig 1)

strains incubated in CO_2 atmosphere and in air. The susceptibility to penicillin G of 44 per cent of the strains examined was the same whether they were incubated in air or CO_2 atmosphere (quotient = 1). The corresponding figures for penicillin V and ampicillin were 33 and 10 per cent respectively. In 90 per cent of the strains the IC_{50} of ampicillin was thus higher when the strains were incubated in CO_2 atmosphere.

Haemophilus Parainfluenzae

By way of comparison the susceptibility of 25 *Haemophilus parainfluenzae* strains isolated from the throat of healthy adults were examined. The variation of the results with the type of ambient atmosphere, i.e. air or CO_2 atmosphere, was essentially the same as that found in cases of *Haemophilus influenzae* strains.

It should however be observed that the *parainfluenzae* strains were much less susceptible to penicillin V than the *influenzae* strains. Only about 50 per cent of the *parainfluenzae* strains were inhibited by 4.8 µg penicillin V/ml (MIC on incubation in air) compared with 95 per cent of the *influenzae* strains. The MIC of one of the *parainfluenzae* strains was 38 µg/ml. The species differed only slightly from one another in susceptibility to penicillin G and ampicillin.

Variation of the Results of the Tests

Table 3 gives the results of 10 determinations of the susceptibility of the two reference strains tested on different occasions.

The MIC of penicillin G and ampicillin sometimes varied by two dilution steps, that of penicillin V by only one. The largest value of the standard deviation was found to be 68 per cent of mean, the lowest 20 per cent. The standard deviation of MIC was larger than that of IC_{50} in 7 of the calculations, that of IC_{50} being larger than that of MIC in 5 of the calculations. Since the results of incubation in CO_2 atmosphere and in air were compared on the basis of determinations made on one and the same occasion, the technical errors involved in such comparisons were smaller than those to be involved if determinations were made on different occasions.

DISCUSSION

The ratio between MIC and IC_{50} varies with the variation of the susceptibility of the individual bacteria in the strain to the antibiotic studied. In the *H. influenzae* strains studied the quotient between MIC and IC_{50} of all three antibiotics ranged from 1.4 to about 3.0 whether the strains were incubated in air or in CO_2 atmosphere. The ratio between MIC and IC_{50} was thus relatively constant, which showed that the strains were fairly homogeneous in this respect. The IC_{50} and MIC were therefore probably equally good measures of the susceptibility of

the strains *Reyn et al* reported similar results obtained by determination of the susceptibility of gonococci to penicillin G (1963) They pointed out that the situated may be different if the strains of bacteria to be examined are heterogeneous in this respect

The reading of the IC_{50} is more subjective than the reading of the MIC The results obtained in the present investigation showed that the error of the method was not decreased by determination of the IC_{5} (Table 3) Grading of the growth by IC_{50} however means that four values can be obtained for each concentration of the antibiotic compared with one value if the MIC is determined This was the only advantage of determination of the IC_{50} over determination of MIC

The susceptibility of several of the strains was found to differ with the type of ambient gases This applies in particular to susceptibility to ampicillin The difference in the distribution of the strains according to susceptibility to ampicillin with the type of gaseous environment was thus greater than that found for penicillin G and V The lowest concentration (MIC) of ampicillin inhibiting all strains incubated in air however also inhibited all strains incubated in CO atmosphere which might mean that the least susceptible strains were affected less than the others in the presence of CO On the other hand the lowest concentration of penicillin G and V that inhibited all strains incubated in CO atmosphere was twice as high as in air (Fig 1)

Many *H influenzae* strains give growth of larger colonies when incubated in CO atmosphere than when incubated in air especially in primary cultures It is however hardly likely that an increased rate of growth *per se* decreases the susceptibility of the bacteria to penicillin in the dilution test as the penicillins have an effect only on actively growing bacteria It is not known how penicillin V and ampicillin differ from penicillin G in mode of action (*Stewart* 1965) In one respect there appears to be a difference between penicillin G and V on one hand and ampicillin on the other on incubated in CO atmosphere the susceptibility to ampicillin was found to be reduced in a number of strains significantly higher than the number of strains in which the susceptibility to penicillin G and V was reduced

It might be mentioned in this connection that a gaseous environment with about 8 per cent CO has been demonstrated in the middle ear in patients with tubal occlusion (*Ingelstedt et al* 1969)

By way of comparison a survey is given of the results of determinations of the susceptibility of *H influenzae* strains to penicillin G penicillin V and ampicillin found by various investigators (Table 4) Only materials consisting of numbers of strains sufficiently large to be regarded as representative were included The material of *Barber & Waterworth* was however included because it appears to be the only one in which all three penicillins were examined (1962)

As to the susceptibility to penicillin G the agreement between the results of the investigations made on solid media including the results

TABLE 4

Published Results of Determinations of Sensitivity of Haemophilus influenzae to Penicillin G, Penicillin V and Ampicillin

Authors	Number of strains	Determination made	Gaseous environment	MIC (μg/ml) pc G	pc V	amp
Finland & Wilcox 1950	30	On haematin agar		0 2 1 25		
Tunevall 1951	53	On Levinthal agar		0 3 2 4		
Mulder et al 1952	187	On Levinthal agar		0 3–4 5		
Love & Finland 1954	90	On haematin agar		0 2–3 1		
Hirsch and Finland 1960	76	On haematin agar	reduced oxygen gas	0 2 6 3		
Goslings et al 1961	30	On Levinthal agar		0 3 2 4	0 6–19 2 § †	
McCarthy et al 1961	37	On haematin agar	CO	0 2 6 3	1 6–10	
Barber & Waterworth 1965	15	On haematin agar		0 2–4 0	2 0 16 0	0 125–0 5
Kohn et al 1967	100	In brain heart infusion broth		0 19–12 5		0 095–0 78
Present investigation	80	On haematin agar	8–10 % CO_2	0 6–4 8	1 2–19 2	0 15 0 60
			air	0 3–2 4	1 2–9 6	0 15–0 60

Corrected from I U to μg § six strains ≧ 19 2 μg/ml † 25 strains examined
Gaseous environment not stated

in the present investigation was found to be better than that apparent from Table 4 in that 96 per cent of the strains in *Finland's & Wilcox's* material were inhibited by 3.1 µg/ml (1950).

As to the susceptibility to ampicillin the agreement was good between the results in the present investigation and those reported by *Barber & Waterworth* (1962) and by *Kahn et al.* (1967) though the last mentioned investigation was carried out in liquid medium. There was also good agreement with the general experience that ampicillin is more active *in vitro* against *H. influenzae* than is penicillin G which is in turn more active than penicillin V. Inhibition of all strains in the present investigation (MIC on incubation of strains in air) required four times as high a concentration of penicillin G as of ampicillin and four times as high a concentration of penicillin V as of penicillin G. On calculation of the corresponding IC_{50} the concentration of penicillin G was 3.3 times as high as that of ampicillin and the concentration of penicillin V 4.7 times as high as that of penicillin G.

As to the susceptibility to penicillin V, *McCarthy et al.* (1961) reported some strains with MIC ≧ 25 µg/ml and *Goslings et al.* (1966) reported MIC ≧ 19.2 µg/ml for as many as 24 per cent of the strains. In the present material all strains were inhibited by 9.6 µg/ml when incubated in air. On incubation in CO_2 atmosphere 95 per cent of the strains were inhibited by this concentration. The strains in the material of *Goslings et al.* (1966) were isolated from patients with chronic bronchitis, i.e. from patients who had probably been treated with antibiotics, possibly on repeated occasions. This in contrast with the strains in the present material, which were isolated from discharge from the ears of otherwise healthy children with acute otitis media. *McCarthy et al.* (1961) do not mention the types of infections of the patients from whom they isolated their strains. The discrepancy in susceptibility to penicillin V between the two above mentioned materials and the present material need not be due to methodological differences because if it were the results obtained in the determination of the sensitivity of penicillin G should also have been different. The discrepancy might instead mean that the *H. influenzae* strains isolated from patients with chronic bronchitis are either primarily less susceptible to penicillin V than those strains that produce acute otitis media in children or that they have become less susceptible to penicillin V owing to previous treatment with this antibiotic. The latter possibility would mean that penicillin V differs from ampicillin and penicillin G in this respect. No increase in resistance *in vivo* of *H. influenzae* to ampicillin and penicillin G has been demonstrated (*Goslings et al.* 1966a, *Hirsch & Finland* 1960, *Stewart* 1963a).

Like the *H. influenzae* strains the *H. parainfluenzae* strains were more sensitive to ampicillin than to penicillin G and least susceptible to penicillin V. Inhibition of all *parainfluenzae* strains (MIC on incubation in air) required 4 times as high a concentration of penicillin G

as of ampicillin and as many as 8 times as high a concentration of penicillin V as of penicillin G The present investigation showed that *H influenzae* can differ widely *from H parainfluenzae* in susceptibility to penicillin V This difference is probably less prominent if the *influenzae* strains are isolated *from patients with chronic bronchitis* Also clinically there is a difference between the two species regarding the genesis of acute otitis media Among some 600 *haemophilus* strains isolated from discharge from the ear in our laboratory during a three year *period* only *two parainfluenzae* strains have been found

SUMMARY

The susceptibility *in vitro* of 80 *Haemophilus influenzae* strains isolated from children with acute otitis media were studied with view to their susceptibility to penicillin G penicillin V and ampicillin using the agar plate dilution method The strains were incubated in air and in 8–10 per cent CO atmosphere

On incubation in CO-atmosphere the minimum inhibitory concentration (MIC) and the 50 per cent inhibitory concentration (IC_{50}) of many strains were higher especially in the determination of their sensitivity to ampicillin

The MIC of all three antibiotics was sometimes four times as high when the bacteria were incubated in CO_2 atmosphere as when they were incubated in air

In a comparative study of 25 *parainfluenzae* strains isolated from the throat of healthy adults several of these strains were less susceptible to these antibiotics than the *influenzae strains* especially to penicillin V

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TABLE 1

In vitro Induction of Resistance in Candida albicans *Candida parapsilosis Candida tropicalis Candida guilliermondii Torulopsis glabrata and Torulopsis famata*

	Antimycotic	Strain	Min I C sensitive strain	Max G C sensitive strain	Max G C resistant strain	Index for induced resistance	No of passage before maximal resistance obtained
	Nystatin µg/ml						
1	Candida albicans	41665 I	20	10	20	2	22
2	*Candida albicans*	8524 A II	20	10	20	2	23
3	Candida albicans	13539	20	10	20	[illegible]	28
4	Candida parapsilosis	3524 A I	40	20	100	5	19
5	Candida parapsilosis	11598	30	20	50	2 5	26
6	Candida tropicalis	[illegible]	*30*	*20*	*30*	1 5	21
7	Candida guilliermond	H 7546	30	20	100	5	21
8	Torulopsis glabrata	A 21113	15	10	100	10	22
9	Torulopsis famata	H 5745	30	20	60	3	13
	Amphotericin B µg/ml						
1	Candida albicans	41665 I	5	2 5	2 5	1	15
2	Candida albicans	8524 A II	5	2 5	10	4	15
3	Candida albicans	13539	5	2 5	2 5	1	15
4	Candida parapsilosis	3524 A I	10	5	1000	200	13
5	Candida parapsilosis	11598	5	2 5	1000	400	12
6	Candida tropicalis	I F 6591	5	2 5	*250*	*100*	15
7	Candida guilliermond	H 7546	–		–	–	–
8	Torulopsis glabrata	A 21113	5	2 5	1000	400	8
9	*Torulopsis famata*	H 5745					
	Trichomycin unit /ml						
1	Candida albicans	41665 I	20	10	40	4	25
2	*Candida albicans*	8524 A II	*20*	*10*	40	4	31
3	Candida albicans	13539	5	2 5	10	4	12
4	Candida parapsilosis	3524 A I	10	20	150	7 5	10
5	Candida parapsilosis	11598	[illegible]	10	100	10	24
6	Candida tropicali	I F 6591	10	5	10	2	12
7	Candida guilliermond	H 7546	10		200	40	5
8	Torulopsis glabrata	A 21113	20	10	40	4	1
9	*Torulopsis famata*	H 5745	5	2	10	4	11
	Polymyxin B µg/ml						
1	Candida albican	41665 I		–			–
2	Candida albicans	8524 A II					
3	Candida albicans	13539	500	250	1000	4	6
4	Candida parapsilosis	3524 A I				–	
5	Candida parapsilosis	11598	250	100	1000	10	6
6	Candida tropicalis	I F 6591	*500*	*250*	*1000*	4	6
7	Candida guilliermond	H 7546	250	100	750	7 5	4
8	Torulopsis glabrata	A 21113					
9	Torulopsis *famata*	H 5745	100	50	250	5	6

Min I C (= minimal inhibitory concentration) i e the lowest concentration of antimycotic producing complete inhibition

Max G C (= maximal growth *concentration*) i e the maximal concentration of antimycotic in which obvious growth could be seen over the whole plate

Index figure i e the ratio between the Max G C (= maximal growth concentration) of the resistant strain to any antimycotic and the parent strain

resistant strain and the Max G C for the original strain Finally the last column shows the number of passages used before maximal resistance was obtained

It will be seen from the table that resistance to *nystatin* was induced in all the candida strains to an extent of 1 5 to 10 times the original resistance while the resistance induced in the torulopsis strains was 3 to 10 times the original resistance

It was difficult to induce resistance to *amphotericin B* in strains of *Candida albicans* It was possible in one strain to induce only four fold resistance while no resistance could be induced in the two other strains High resistance could be induced in the two *Candida parapsilosis* strains (index 200 and 400) The resistance to amphotericin B was over 100 times the original resistance in the *Candida tropicalis* strain and 400 times that in the *Torulopsis glabrata* strain The strains of *Candida guilliermondii* and *Torulopsis famata* showed natural resistance to amphotericin B

Four fold resistance to *trichomycin* could be induced in the *Candida albicans Torulopsis glabrata* and *Torulopsis famata* strains In the *Candida parapsilosis* strains the index figures for induced resistance were 7 5 and 10 An increase in resistance of twice the original resistance was induced in the *Candida tropicalis* strain while the increase was forty fold in the *Candida guilliermondii* strain

Resistance to *polymyxin B* was induced in five out of the nine strains two *Candida albicans* one *Candida parapsilosis* and one *Torulopsis glabrata* showing natural resistance As regards the remaining strains the increase in resistance to polymyxin B was four to ten times the original resistance

The rate at which resistance to the various antimycotics developed can be seen from the right hand column in Table 1

It was possible in all strains to demonstrate resistance to *nystatin* after 20 passages and in some cases with fewer *e g* Strain 9

Strains 7 and 9 showed natural resistance to *amphotericin B* As to the other strains resistance could be demonstrated after 15 passages (except for Strains 1 and 3) In several strains fewer passages sufficed (*e g* Strains 4 5 and 8)

As regards all strains it was possible to demonstrate resistance to *trichomycin* after 20 passages For several strains fewer passages were necessary *e g* Strains 3 4 6 8 and)

Strains 1 2 4 and 8 showed natural resistance to *polymyxin B* Resistance to that drug could be demonstrated after five passages in the case of Strains 3 5 6 and 9 and after only three passages in the case of Strain 7

It was easiest to induce resistance to polymyxin B more difficult to amphotericin B and most difficult to nystatin and trichomycin

Cross Resistance

The results of cross resistance experiments are shown in Table 2. It will be seen that the nystatin resistant cells of *Candida tropicalis* became five times more resistant to amphotericin B than the original sensitive cells and that nystatin resistant cells of *Torulopsis glabrata* developed a forty fold resistance to that drug.

Correspondingly amphotericin B resistant cells of *Candida tropicalis* developed a resistance to nystatin that was twice as large as that of the originally sensitive strains; in the case of *Torulopsis glabrata* it was eight times larger than the original sensitive strains.

TABLE 2
Cross Resistance

Strain No				Increased resistance to amphotericin B	Increased resistance to nystatin
6	Candida tropicalis	PF 1591	Nystatin	5 ×	
8	*Torulopsis glabrata*	A 21113	*resistant cells*	40 ×	
6	Candida tropicalis	PF 1591	Amphotericin B		2 ×
8	Torulopsis glabrata	A 21113	resistant cells		8 ×

Other Investigations

During the first phases of resistance development particularly large colonies more chalk white than those generally seen in the culture concerned were sometimes observed both on Szybalski gradient plates and during sensitivity determinations. At that stage of resistance development cells from the large colonies showed greater resistance to the antimycotic than the culture as a whole. With increasing concentrations of antimycotic the tendency to the formation of large colonies decreased gradually as the resistance developed. The biggest change in this respect was seen in the case of Strain 6 *Candida tropicalis* during culture on medium containing amphotericin B. Giant colonies were observed both on Szybalski gradient plates and on resistance plates with an amphotericin B concentration ranging between 25 and 75 μg/ml medium after culture at 37 °C for five days. These had an opaque undulating mycelium covered surface and a diameter about ten times greater than the normal average colony diameter of the strain in question. The colonies were spread uniformly over the plate often lying next to normal colonies with smooth convex surface (Figs 1 and 2). If the sensitive original strain and the strain with maximum resistance were inoculated together on Sabouraud's maltose agar without antimycotic the difference in growth was evident. The colonies of the sensitive strain were considerably larger than those of the resistant strain.



Fig 1

Amphotericin B gradient plate (50 $\mu g/ml$ in upper layer) inoculated with [illegible] *tropicalis* (Strain 6 PF 6591) Culture at 37 C for 5 days [illegible] can be seen next to a "giant" colony

The more stable the induced resistance the more inoculations on medium without antimycotic were necessary before the growth of the resistant strain corresponded to that of the original sensitive strain. When a higher grade of resistance was achieved the [illegible] was lost and the colonies seemed duller and dryer.

No difference as regards the stainability of sensitive and resistant strains could be seen in Gram stained smears.

Biochemical Changes

The ability to ferment and assimilate carbon sources and to assimilate nitrogen was examined with both the original and the resistant cells.

No changes were observed in the biochem[illegible] ant strains other than those which can be ex[illegible] of growth of such strains.

DISCUSSION

The experimental results show that [illegible] resistance to nystatin amphotericin B [illegible] in certain yeasts of the species candida [illegible]



Fig [illegible]

Same area as marked by the arrow in Fig 1 but with magnification about ten times

Furthermore it was found that despite the use of Szybalski's gradient plate technique the degree of resistance developed was low. These findings seem to be in agreement with the few published reports concerning the *in vitro* development of resistance in *Candida albicans* and other yeasts (*Casida & McCoy* 1952 *Perry & Ulrich* 1955 *Stout & Pagano* 1956 *Forni* 1957 *Littman et al* 1958 *Jones & Peacock* 1959 *Sorensen et al* 1959 *Hebeka & Solotorovsky* 1962 1965) They also agree with the observation of *in vitro* development of resistance in *Candida albicans* isolated from two patients under treatment with antimycotics (*Bodenhoff* 1968 a)

It seems therefore justified at present to assume that it is generally difficult to induce resistance to antimycotics in yeasts or at any rate to induce any high degree of resistance. The previously observed development of marked resistance in *Cryptococcus neoformans* (*Bodenhoff* 1968 b) must thus be considered an exception

To induce resistance to nystatin and amphotericin B was somewhat easier than expected judging by the published reports of efforts to induce resistance in candida strains. The use of the Szybalski's gradient plate technique is probably the explanation of this since concentration intervals can thus be avoided. This concurs with the observation of *Littman et al* (1958) that it is easier to induce resistance in candida by the use of small concentration intervals and many inoculations over

longer periods Marked increase in resistance often takes places in the first and/or last passages while intermediate passages are sometimes interrupted by stages in which no resistance increase can be seen It is also evident from the experiments that when once a certain resistance has been induced *it is often possible to induce greater resistance* more quickly It has been demonstrated in this study that the same maximal resistance is achieved in the same strain after the same number of passages if the same technique is used

Resistance to a certain antimycotic is not achieved equally easily by the different yeasts This applies not only to the different types but also to different strains within the same type An example of this is the difference in index sizes for the induced resistance to amphotericin B This finding is in agreement with *Littman et al* (1958)

Neither is resistance to different antimycotics acquired with equal ease by a certain strain In the present study resistance to nystatin was induced in all nine strains but only to a low degree and after many passages This agrees with the findings obtained by *Donovick et al* (1955) *Stout & Pagano* (1956) *Littman et al* (1958) and *Hebeka & Solotorovsky* (1965) who could not demonstrate resistance to nystatin in any of the strains they used Induction of resistance to nystatin is made difficult by the fact that the nystatin concentration in the suspension used is not known exactly and also by the lability of the drug

In agreement with reports in the literature (*Stout & Pagano* 1956 *Littman et al* 1958 *Hebeka & Solotorovsky* 1965) hi amphotericin B could not be induced in *Candida albica* the attempts to induce resistance failed Higher resi achieved with the other yeasts used in the study

Cross resistance to polyene antimycotics was demon *& Pagano* (1956) *Littman et al* (1958) and *Sorens Hebeka & Solotorovsky* (1965) found that cells of (made resistant to amphotericin B showed cross resist but not to nystatin Furthermore they found that cell to candidin also showed increased resistance to ampho to nystatin In the present stu y one strain of *Candi* one strain of *Torulopsis glabr* a were examined for cr nystatin and amphotericin B The experiments were times and each time cross resi ance to the polyene a found in both strains

In agreement with *Hebeka* ... *vsky* s obse changes in colony morph ... uced rate of ... demonstrated in ... istant cultures as compared to sensitive cultures while no changes in the ... inability of resistant and sensitive cells could be found by means of Gram staining

SUMMARY

Seven strains of candida and two of torulopsis were exposed by means of Szybalski's gradient plate technique to increasing concentrations of nystatin amphotericin B trichomycin and polymyxin B

In the majority of the strains resistance to the antimycotics named could be induced With a few exceptions the degree of resistance was low The resistance developed quickest to polymyxin B and quite quickly to amphotericin B but only after relatively many passages resistance developed to nystatin and trichomycin

Cross resistance to nystatin and amphotericin B was found in *Candida tropicalis* and *Torulopsis glabrata*

Changes in colony morphology and reduced rate of growth of the resistant strains were observed

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The Institute of Medical Microbiology University of Lund
Lund Sweden

ENDOTOXIN INDUCED CYTOTOXICITY OF RABBIT SERUM

By

HANS FRITZ and ERIK NORDENFELT

Received 10 x 68

The effect of endotoxin on tissue cultures has been the subject of previous studies (*Mesrobeanu et al* 1960 *Bergman & Nilsson* 1963 *Kessel* 1965) But no investigation appear to have been made of the effect of tissue fluids in experimental endotoxinaemia on cell cultures The mechanism *in vivo* of the tissue damage following endotoxinaemia is debatable (see *inter alia Landy & Braun* 1964) The effect on cell cultures of tissue fluids in experimental or clinical endotoxinaemia may contribute to the understanding of the way in which endotoxins produce their effect *in vivo* Such investigations may also suggest an approach for the development of laboratory methods for the diagnosis of endotoxinaemia It was previously reported (*Fritz & Nordenfelt* 1967) that characteristic cytotoxic changes appeared in cultures of monkey kidney cells and human amniotic cells when serum from rabbits that had received intravenous injections of endotoxin was added to the nutrient medium Addition of endotoxin instead of such serum produced no demonstrable toxic effect The present paper gives a detailed report on that finding

MATERIAL

Forty rabbits weighing 2000–4800 grams and obtained from local breeders The *endotoxins* were prepared according to Westphal from *Salmonella abortus equi* (Difco 3127) and *Escherichia coli* (O 111 B4 Difco 3192) Endotoxin from *Proteus mirabilis* was kindly supplied by Prof C Weibull University of Lund and prepared by him according to Westphal (*Westphal* 1956 *Westphal et al* 1958) Endotoxin from *Salmonella enteritidis* (Ribi 6-795) was kindly supplied by Prof E Ribi Hamilton Montana, U S A and prepared according to *Ribi et al* (1961)

Cell cultures in glass culture tubes were prepared from monkey kidneys and obtained from Statens Bakteriologiska Laboratorium Stockholm and from human amniotic tissue and prepared according to *Lavelle* (1956) The nutrient medium consisted of Eagle's medium for monkey kidney cells and Parker's medium for human amniotic cells Two per cent calf serum was included in both media

This investigation was supported by grants from *Kungl Fysiografiska Sällskapet i Lund* and *the Medical Faculty University of Lund*



Fig 1 a

A culture of monkey kidney cells in a monolayer incubated with *normal rabbit* serum in Eagle s medium for 2 days and photographed through the culture tube glass Intracellular structures are not visible but the elongated spool shaped form of most cells is demonstrated

METHODS

50–200 μg of endotoxin dissolved in 1 ml of di tilled water was injected into an ear vein Before and at various intervals *after the injection* blood samples were obtained from a vein of the opposite ear or by heart puncture whereby the rabbits were bled to death Xylol was used for vasodilatation of the ear The serum was *separated off* within *2 hours and stored at* —20 C

Experimental series I includes 18 rabbits Fifteen rabbits were given 200 μg of endotoxin intravenously from *Salmonella abortus equi* and for control 3 rabbits received 1 *ml of distilled water* intravenously *Out of the 18 animals 6 (including* the controls) were killed by bleeding 2 hours after the injection 6 after 4 hours and the remaining 6 after 7 hours The sera were tested on monkey kidney and amniotic *cells* Two rabbits died within 2 hours and 4 hours respectively after *the injection of endotoxin*

Experimental series II was carried out on 27 rabbits Thirteen of these received endotoxin from *Salmonella abortus equi* in a dose of 50 100 or 200 μg Nine rabbits were given 200 μg of endotoxin from other enterobacteria 5 from *E coli* 2 from *Proteus mirabilis* and 2 from *Salmonella enteritidis* Repeated blood samples were obtained at intervals from half an hour to 144 after the injection of endotoxin In all the rabbits the blood vessels of the ear were severely *constricted after the injection* of endotoxin This made it difficult and sometimes impossible to obtain blood samples in all planned intervals Ten of the 22 rabbits died within two days of the injection of endotoxin

The cell cultures were washed once with the respective nutrient media and supplied with 1 ml of fresh medium without added calf serum after *which 0 1 ml of* rabbit serum undiluted or diluted with saline was added Each serum was tested on 3 culture tubes After 24 hours incubation at +37 C *the medium* was replaced



Fig 1 b

Monkey kidney cell culture incubated for 2 days with serum from rabbits pretreated with endotoxin A dark granulati n of the cytoplasm with the nucleus appearing as a hollow space is easily seen without any preceeding staining procedure The cells are swollen and not as elongated as in the normal culture

b fresh medium containing calf serum in a concentration of 2 per cent The cell cultures were inspected at least every other day in the culture tube under the microscope and the cytotoxic effect in form of cytoplasmic changes was noted

In control experiments monkey kidney cells and amniotic cells respectively were incubated ith 200 µg of endotoxin in 0 1 ml of distilled water per culture tube Rabbit serum pre incubated for 4 hours at +37 C with 200 µg of endotoxin per ml was added to another series of culture tubes Monkey kidney cells and amniotic cells were also pre incubated for 24 hours with 100–200 µg of endotoxin before addition of rabbit serum from blood collected before respectively after the injection of endotoxin into the animal

RESULTS

Experimental Series I

Effect of Endotoxin from Salmonella Abortus Equi

Addition to the tissue culture tube of sera from blood samples obtained from 3 of the 18 rabbits before the injection of endotoxin resulted in mild shrinkage and slight peripheral granulation of the monkey kidney cells These changes were classified as non characteristic degeneration Sera from blood samples obtained from the other 15 rabbits before the injection of endotoxin produced no visible effect on the cell cultures

The cell cultures incubated with sera from blood samples obtained after the injection of endotoxin regularly showed distinct changes characterized by granulation of the cytoplasma of the cells with the nucleus appearing as a defect (Fig 1 and 2) The normally spindle shaped cells appeared to be swollen and oval In most cultures these changes occurred within 24 hours incubation and were fully developed within 48-72 hours These changes were regarded as a cytotoxic effect

The sera from the 3 rabbits used as controls and obtained after the injection of distilled water had no cytotoxic effect

Cytoplasmic granules in cells incubated with serum from blood samples collected after the injection of endotoxin were readily stained with Sudan III and could easily be extracted with fat solvents such as alcohol and ether

Experimental Series II

Among the sera from blood samples obtained before the injection of endotoxin from the 22 rabbits used in this series 18 had no demonstrable effect on monkey kidney cells while 3 produced non characteristic degeneration of the same type as in experimental series I and described above These 3 sera had no demonstrable cytotoxic effect on amniotic cells One of the 22 sera had a cytotoxic effect on both monkey kidney cells and amniotic cells The effect resembled that to occur after incubation of cell cultures with serum from blood samples collected after the injection of endotoxin The rabbit in question showed no signs of disease

Effect of Endotoxin from E Coli Proteus Mirabilis *and* Salmonella Enteritidis

Endotoxin in a dose of 200 μg was injected into 6 rabbits on one and the same occasion In 2 experiments the endotoxin emanated from *E coli* in 2 *from Proteus mirabilis* and in 2 *from Salmonella enteritidis* One rabbit of each pair was bled to death 4 hours and 24 hours respectively after the injection Sera from all the rabbits had the same cytotoxic effect as sera from the rabbits that had received endotoxin from *Salmonella abortus equi* Sera from the 2 rabbits that had received endotoxin from *E coli* were tested also on human amniotic cells and with the same results

Control Studies

The endotoxins used had no cytotoxic effect when incubated with cell cultures Neither did pretreatment of the cell culture with endotoxin increase or reduce their sensitivity on subsequent incubation with rabbit serum from blood samples collected after injection of

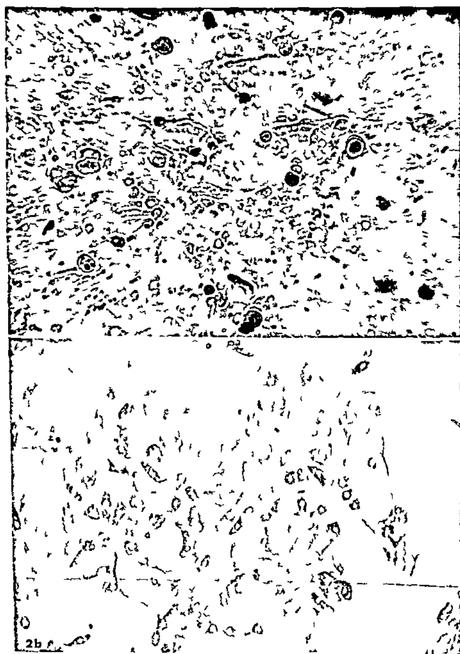


Fig 2

A cult[illegible] f human a[illegible] t[illegible] ell incubat[illegible] [illegible] th normal rabbit serum (a) and ral bit serum [illegible] btaine[illegible] after inj[illegible] t on of end toxin (b)

endotoxin Normal rabbit serum incubated with endotoxin was not cytotoxic

Appearance and Duration in Vivo of the Cytotoxicity of the Sera

Each of 5 rabbits was given 200 µg of endotoxin from Salmonella abortus equi and blood was sampled on 3-5 occasions From 2 rabbits blood samples were obtained already half an hour after the injection of endotoxin and no cytotoxic effect of the serum was found From 2 out of 3 rabbits sera proved cytotoxic 1 hour after the injection Between 4 and 48 hours the sera were always cytotoxic and toxic sera could from two rabbits be obtained up to 60 and 72 hours respectively after the injection of endotoxin

One out of 3 rabbits survived an injection of 200 µg of endotoxin from *E coli* From this rabbit 6 blood samples were collected at 24 hour intervals after the injection The first 4 samples i e up to 96 hours after the injection of endotoxin had a cytotoxic effect while the last 2 had no demonstrable effect

Effect of Different Doses of Endotoxin

Five rabbits were given 100 µg of endotoxin each In all of these serum from blood samples collected 16-24 hours after the injection proved cytotoxic In 4 of these 5 rabbits blood samples were collected also 4 hours after the injection of endotoxin in 3 of these the serum then had a cytotoxic effect Two rabbits were given 50 µg of endotoxin Eight hours after the injection serum from one of these proved cytotoxic

Serum Content of Cytotoxic Factors

The changes in the cell cultures were more or less pronounced with respect to the density of the granulation in the cells and the number of granulated cells The severity of the changes sometimes varied from one to another of the 3 culture tubes incubated with the same serum The cytotoxic effect of the serum also varied with the cells originating from different monkeys The variation was however not so wide that a given serum had a cytotoxic effect on one cell culture but not on another

Using 4 rabbits two of which received 100 and two 200 µg of endotoxin we tried to assess the content of cytotoxic factors in the serum by the dilution technique Blood samples collected 1 4 24 and 42 hours after the injection of endotoxin were included All the sera could be diluted to a final concentration of 1 30-1 40 without disappearance of their cytotoxic effect In no instance could the serum be diluted more than 1 50 without loss of its cytotoxic effect

Ratio between Fat Content and Cytotoxic Effect of Serum

Since the changes in the cell culture might possibly be related to an increase of the fat content of the serum caused by endotoxin the concentration of cholesterol and that of triglycerides were determined in 20 samples In most rabbits the fat content increased successively during the first 24-48 hours after the injection of endotoxin Samples collected 24 hours after the injection of endotoxin were regularly macroscopically fatty and had the highest content of cholesterol and triglycerides But cytotoxic sera from some of the samples obtained 2-4 hours after the injection of endotoxin contained less cholesterol and/or triglycerides than serum from samples collected before the injection of endotoxin

Properties of the Cytotoxic Factor in Serum

Sera from rabbits that had received an injection of endotoxin still had a cytotoxic effect after being heated at 56 C for 30 minutes The cytotoxic effect persisted after dialysis of the sera with distilled water physiological saline or 0 5 M phosphate buffer at pH 7 4 Normal rabbit sera did not turn cytotoxic after dialysis The cytotoxic effect persisted also after the sera had been thawn and frozen 5-6 times and after storage for one week at room temperature

In an attempt to elucidate the specificity of the cytotoxic factor in serum appearing after i v injection of endotoxin we tested sera from rabbits in which shock reactions had been provoked In one rabbit anaphylactic reactions were produced by repeated injections of albumin Serum from blood samples obtained in association with these reactions had no cytotoxic effect In 3 rabbits shock was produced by bleeding Repeated serum samples 1-24 hours thereafter did not show cytotoxic effect except in one case where the serum from a blood sample collected 24 hours after the shock was produced proved cytotoxic

DISCUSSION

Mesrobeanu et al (1960) found that endotoxin prepared according to *Boivin & Mesrobeanu* (1938) caused hypertrophy and fatty degeneration of the cells in tissue cultures of human embryo embryo chick and monkey testicle cells Embryonic chicken tissue was the most susceptible The cytotoxic effect could be produced by 10-100 μg of endotoxin per cell culture tube On cultures of HeLa cells however these authors found no cytotoxic effect of endotoxin In our experiments with monkey kidney cells and human amniotic cells incubated with endotoxin prepared according to Westphal and Ribi respectively the endotoxin was not found to have any visible cytotoxic effect not even when the cells were incubated with 200 micrograms of endotoxin per culture tube

In 1962 *Mesrobeanu et al* published an examination of the effect of neurotoxin or so called termolabile endotoxin on human embryonic cells and on tumor cells and they found cytoplasmic changes characterized by pronounced fatty degeneration It seems that the changes produced by the neurotoxins morphologically resemble those produced by sera from endotoxin treated rabbits in the present study

After intravenous injection into rabbits endotoxin soon leaves the circulation (*Carey et al* 1958 *Herring et al* 1963) The endotoxin is taken up mainly by the reticulo endothelial cells in the liver A sublethal dose i v of radioactively labelled endotoxin disappears almost completely from the circulation within 30–45 minutes It is therefore probable that the cytotoxicity of rabbit serum demonstrated in our experiments is not due to the endotoxin *per se* but rather to cytotoxic substances released into the circulation or generated by the endotoxin injected

In vivo tissue lesions secondary to endotoxins are mainly caused by inoxin Endotoxinaemia is followed by severe vasoconstriction and/or occlusion of small vessels because of increased sensitivity to catecholamines or thrombosis coagulation or aggregation of blood cells (*Gerber* 1936 *Thomas* 1956 *Hardaway* 1963 *Fine* 1964 *Lee* 1964 *Fritz et al* 1965 and others) Cellular substances thereby released may perhaps have a cytotoxic effect on tissue cultures

It is well known that endotoxin injected into the rabbit can produce hyperlipaemia and hypercholesterolaemia (*Hirsch et al* 1964 *Lasch* 1968) As an effect of catecholamines the lipid metabolism may be markedly changed (*Carlson & Liljedahl* 1963) The highest concentration of cholesterol in the serum after an injection of endotoxin is attained usually after 16 30 hours In our experiments rabbit serum collected already 2–4 hours after the injection of endotoxin had a cytotoxic effect when serum showed a normal content of cholesterol and triglycerides The concentration of free fatty acids on the other hand shows a temporary increase starting already within one hour after an injection of endotoxin and decreases after a few hours (*Hirsch et al* 1964 *Lasch* 1968) It therefore appears improbable that changes in the cell cultures are produced by a high concentration in the serum of cholesterol triglycerids or free fatty acids The relation of the cytotoxicity to the fatty acid composition of serum after an injection of endotoxin is receiving further attention

It is known that acid phosphatase glucoronidases lysozyme and proteases are released to the circulation after the injection of endotoxin (*Martini* 1959 *Weissman & Thomas* 1962) An investigation of the effect of such enzymes on cultures of monkey kidney cells and human amniotic cells is in progress

In a few experiments made on the specificity of the cytotoxic factor with provoked shock reactions one interesting finding was made In prolonged shock caused by bleeding there is an escape of endotoxin

from the intestine causing endotoxinaemia (*Fine* 1954 1959) In agreement with this we found in one rabbit in shock caused by repeated bleeding a serum sample collected after 24 hours which proved cyto toxic

SUMMARY

Intravenous injection into rabbits of 100–200 μg of endotoxin from *Salmonella abortus equi* was regularly followed by the appearance of a cytotoxic effect of serum The cytotoxic changes demonstrated in monkey kidney cells and human amniotic cells were characteristic and dominated by an accumulation of fat in the cytoplasm Administration of endotoxin from *E coli Proteus mirabilis* and *Salmonella enteritidis* also resulted in cytotoxicity of the serum The cytotoxic effect of the sera was demonstrable in samples collected between one hour and 96 hours after the injection of endotoxin It persisted after heating of the serum at +56 C and after dialysis of the serum Incubation of normal rabbit serum with endotoxin *in vitro* did not make the serum cytotoxic

Addition of endotoxin alone to the cell cultures had no cytotoxic effect

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Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt
University of Oslo Rikshospitalet Oslo Norway

THE SEROLOGY OF *MORAXELLA KINGII*

By

RANDI HOLTH HAUG and S D HENRIKSEN

Received 11 x 68

Only few and rudimentary studies of the serology of organisms belonging to the genus Moraxella have been carried out In the early studies (*Chaine* 1914 *Scarlett* 1915) single strains of different species were compared and found to have different antigens But the possibility that each species might be serologically inhomogeneous was not considered and the results therefore were of little value

Later a few strains of Moraxella species have been included in serological studies of such organisms as *Mima Herellea, Bacterium anitratum Diplococcus mucosus* etc (*Cary et al* 1956 1961 *Mitchell & Burrell* 1964) But the value of these studies is limited by a lack of precise identification of the strains used Thus on the whole our knowledge of the serology of these organisms is very meager

Attempts to trace serological relationships within and between Moraxella species and with other organisms which have been thought to be related to Moraxella will be described in a subsequent paper The purpose of this paper is to report studies of the serology of the newly described species *M kingii* (*Henriksen & Bovre* 1968)

MATERIAL

The strains used in this stud[illegible] a[illegible]e presente[illegible] in Ta[illegible]le 1

METHODS

Immunization *M kingii* strain 530 wa[illegible] grown on tryptos[illegible] agar for 24 h at 33 C in a humid atmosphere and harvested [illegible]n saline On[illegible] half of the suspension was sonicated (MSE Ultrasonic D[illegible]s [illegible]) for 2 × 2 5 min [illegible]s described by *Mitchell & Burrell* (1964 [illegible] centrifuged [illegible] the sediment discarded

Before use th[illegible] [illegible] k[illegible]d for disintegration by microsc[illegible] and for sterility by culture on [illegible] agar The other half of th[illegible] suspension was measured in a Beckman colorimeter at wave length 574 mμ and the transmittance wa[illegible] adjusted to ca 20 [illegible] Equal part[illegible] [illegible]f the[illegible] antigens were mixed and [illegible]mul[illegible]fied [illegible]n Freund's complete adjuvant (Difco) On[illegible] half ml quantities [illegible]ere injecte[illegible] int[illegible]amuscul[illegible]rly into rabbits 3 times at weekly intervals follo[illegible]ed b[illegible] 3 intravenou[illegible] injections [illegible]f antigen without adjuvant on con[illegible]ecutive days starting [illegible]ne we[illegible]k after the l[illegible]st intramuscula[illegible] injection Cardiac puncture with exsangui[illegible]tion [illegible]a[illegible] [illegible]ne 5 days later The [illegible]r[illegible]m was pre[illegible]erved with merthiolate 0 01 p[illegible]r[illegible]ent and stored frozen until used

TABLE 1

Strains Used in the Study

Species	Strains	Isolated by	Received from
Moraxella kingii	5530 A6042 5693(1) 8623 9571 11702 A2471 2941	E O King	CDC
	4177/66 3379/67 3793/67 3994/67 4767/67 6111/67	S D Henriksen	WW
Moraxella osloensis	41990 5873 8292	E O King	CDC
	19118/61	S D Henriksen	WW
	10973	?	SSI
Moraxella nonliquefaciens	672/58 826 4862 3828/60 4378/62 3328/62 2919/66	S D Henriksen	WW
	13385	S D Henriksen	NIFH
	7784	?	NCTC
	17954	R G E Murray	ATCC
	17975	M Piéchaud	ATCC
Moraxella phenylpyruvica	752/52	S D Henriksen	WW
	2863	E O King	CDC
	17958	H Flamm	ATCC
Unclassified[a]	17955	M Piéchaud	ATCC
Moraxella lacunata	9833	?	NCTC
Moraxella bovis	9415	?	NCTC
Acinetobacter calcoaceticus[b]	15150	W Ferguson	ATCC
	15319	F Billing	ATCC
Acinetobacter lwoffi	17985	?	ATCC
	17977	M Piechaud	ATCC
Bacteroides corrodens	4503/67 4665/67 4954/67 59557/67 4672/67 3863/67 3852/67	S D Henriksen	WW
	333/54-55 310A/54-55 310B/54-55	M Eiken	SSI
	61R	L. Reinhold	MLU

[a] the strain resembles *M nonliquefaciens* in most characters but deaminate phenylalanin

[b] *calcoaceticus* appears to be the correct epithet of the organism formerly known as *Acinetobacter* (*Bacterium*) *anitratus*

CDC Communicable Disease Center Atlanta Georgia
WW Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt
NIPH National Institute of Public Health Oslo
NCTC National Collection of Type Cultures London
ATCC American Type Culture Collection Rockville Maryland
SSI State Serum Institute Copenhagen
MLU Martin Luther Universitat Halle/Saale

The strains 3379/67 and 2941 were grown on chocolate agar for 24 h at 33 C in a humid atmosphere The cells were harvested in 0 85 per cent saline the suspensions centrifuged and the sediments resuspended in 0 85 per cent saline The suspensions were injected intravenously into rabbits on 3 consecutive days for 3 weeks with antigen dose starting at 0 25 ml and increasing to 1 ml Cardiac puncture after 5 days and treatment of the sera as described above

Agglutination M kingii and B corrodens were grown on chocolate agar for 24 h at 33 C in a human atmosphere The other organisms were grown on tryptose agar Cells were harvested with 0 2 per cent saline One part of each suspension (antigen *a*) was measured in a Beckman colorimeter wave length 524 mμ and adjusted to a transmittance of c 40 and preserved with merthiolate 0 01 per cent Another part (antigen *b*) was heated to 100 C for 1 h and washed 4 times with saline before resuspending in 0 2 per cent saline Twofold serum dilutions from 1 10 were made with 0 2 per cent saline since some antigens were more stable at this salt concentration Equal volumes of antigen were added to the serum dilutions and the tubes were incubated in a water bath at 37 C for about 20 h. before being read

Absorptions Small portions of serum were absorbed with boilt antigens over night centrifuged and the supernates tested for remaining agglutinins. If the absorption was incomplete it was repeated with new antigen

Immunodiffusion Ouchterlony s technique using Special Agar Noble (Difco) 1 2 g/100 ml of 0 85 per cent saline containing 1 ml of 0 3 per cent methyl orange and 1 ml of 1 per cent merthiolate Arrangements with one central well for immune serum and 6 peripheral wells for antigens were used Heavy suspensions of cells were disintegrated as described under immunization and the supernates used as antigens

RESULTS

All strains of *M osloensis* *M nonliquefaciens* *M phenylpyrouvica* *M lacunata* *M bovis* *A calcoaceticus* and *A lwoffi* and the unclassified strain gave negative reactions in agglutination tests both with *a* and *b* antigens The reactions of *M kingii* and some *B corrodens* strains are shown in Table 2 The remaining strains of *B corrodens* were not agglutinated

TABLE 2

Agglutination of M kingii *and* B corrodens *in Immune Sera against* M kingii

Antigens	Immune Sera 5530 a	5530 b	3379/67 a	3379/67 b	2941 a	2941 b
K A6042	0	640	0	0	80	40
K 5693(1)	0	160	40	1280	20	80
K 8623	0	640	0	0	40	160
K 9571	0	160	0	640	80	640
K 4177/66	0	0	0	640	20	320
K A1702	0	1280	0	20	160	160
K A2471	0	640	0	0	40	0
K 3793/67	0	0	40	2560	0	1280
K 3974/67	0	640	0	0	40	0
K 6111/67	0	0	160	20	160	40
K 4767/67	0	640	20	0	20	0
K 5530	0	1280	0	0	20	640
K 3379/67	0	0	40	2 60	0	320
K 2941	0	0	0	40	80	640
C 4503/67	0	0	40	160	0	0
C 4265	0	0	160	80	0	0
C 333/54 55	0	0	40	0	0	0
C 3862/67	0	0	160	0	0	40
C 3852/67	0	0	160	40	0	0

C = *B corrodens* K = *M kingii* a = unheated antigen b = heated antigen

B corrodens strains 61R, 310B/54–55 4254/67 S9557/67 310A/54 55 and 4672/67 gave entirely negative reactions, likewise all the other organisms shown in Table 1

TABLE 3
Absorption Tests

	Immune Sera			
	3379/67		2941	
	Tested with			
Absorbed with	Homologous antigen	Absorbing antigen	Homologous antigen	Absorbing antigen
2941	1280	0	0	0
3379/67	0		640	0
3793/67	0	0	640	0
4177/67	1280	0	640	0
5530			640	0
5093(1)	40	0	640	0
9571	1280	0	320	0

Two absorptions were needed to remove agglutinins

The *a* antigens gave entirely negative reactions in one serum and only very weak and irregular reactions in the other two sera. With the *b* antigens the serum 5530 gave clearcut results. Seven strains gave strong and firm reactions to approximately the same titres (640 to 1280). Two strains gave weak partial agglutination with the titre 160 and the remaining strains were not agglutinated. The reactions with the other two sera were more confusing with variations of titres and of the strength of agglutination. In order to clarify the meaning of these reactions absorption tests were carried out. The results are shown in Table 3. Three strains which had given titres of 1280 or 2560 with the serum 3379/67 were able to remove all agglutinins reactive with the homologous antigen whereas the two strains which gave titres of 640 only removed the agglutinins reacting with the strains themselves but failed to reduce the homologous titre.

From the serum 2941 only the homologous antigen was able to remove all agglutinins whereas the other strains removed agglutinins reacting with themselves but failed to reduce the homologous titre.

Five strains of *B corrodens* gave weak and low titered reactions in two of the sera. These reactions seem to be of very doubtful significance in particular in view of the tendency of these strains to form unstable suspensions. Six strains gave entirely negative reactions.

In immunodiffusion all *M kingii* strains gave two or three precipitate bands with the sera. These bands showed reactions of identity. Among the antigens prepared from the 11 *B corrodens* strains 6 gave two precipitate bands in the sera 3379/67 and 2941 after 24 h and 5 gave one band after 24 h and a second band after 3 days. In the serum 5530 7 strains gave one band after 24 h. The bands produced by the *B corrodens* antigens appeared to show reactions of identity mutually and with bands produced by *M kingii* antigens.

In order to exclude the possibility that any of these bands could be

due to horse serum antigens from the medium the tests were repeated after absorption of the sera with horse serum but the precipitate bands were not affected

Among the antigens prepared from other Moraxella species and the other organisms listed in Table 1 only a single one prepared from *M osloensis* 8292 produced a single precipitate band whereas the others failed to react

DISCUSSION

The results indicate that cells of *M kingii* carry a thermolabile surface component which blocks agglutination This substance which can be removed by heating to 100 C for 1 h appears to be nonantigenic or of low antigenicity Thus one of the immune sera failed to agglutinate unheated suspensions of any of the 14 strains and in the other sera only weak and irregular reactions were obtained Some of these reactions might be explained as due to incomplete inhibition of agglutination by the blocking surface component whereas other reactions could not be thus explained It seems reasonable to consider these weak and unconvincing reactions as nonspecific

The results obtained with agglutination and absorption tests with heated antigens suggest that the strains could be divided into a limited numer of serotypes Thus 7 out of the 14 strains gave strong and fairly high titered reactions in the serum 5530 Available quantities of this serum were not sufficient for absorption tests Among the 5 strains which gave fairly strong reactions in the serum 3379/67 3 which gave *titres of 1280 or 2560* were able *to remove the agglutinins reacting* with the homologous antigen although more antigen of the heterologous strains than of the homologous strain was needed to exhaust the serum The two strains which gave reactions with the titre 640 failed to remove the homologous agglutinins From the serum 2941 only the homologous strain was able to remove the agglutinins reacting with the homologous antigen

Thus by means of three immune sera 11 out of 14 strains could be assigned to 3 serotypes Among the remaining 3 strains two gave marked cross reactions in two of the sera whereas the third gave only weak reactions in these two sera

The fact that as many as 11 out of 14 strains could be assigned to as few as 3 serotypes suggests that the serology of this species may be fairly str[illegible] but more strains will have to be examined before the number of existing serotypes can be estimated It may be pointed out that there is no correlation between serotype and type of colony or geographical origin since both the main serotypes contained strains in the smooth form as well as in the corroding form and both Norwegian and American strains

The weak and low titered reactions with unheated and heated antigens of some strains of *B corrodens* may be suspected of being non

Acta path microbiol scand 75 648-656 1969

Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt
University of Oslo Rikshospitalet Oslo Norway

SEROLOGICAL STUDIES OF A COLLECTION OF STRAINS OF *MORAXELLA* SPECIES AND RELATED ORGANISMS

By

BARBRO HOLTH HAUG and S. D. HENRIKSEN

Received 11.x.68

Results of studies of the serology of *Moraxella kingii* were reported in a previous paper (*Holth Haug & Henriksen* 1969). This paper deals with additional studies of other Moraxella species.

MATERIAL AND METHODS

The strains used are the same as presented in Table 1 of the preceding paper (*Holth Haug & Henriksen* 1969). *M. kingii* 5530 was used both for agglutination and immunodiffusion; the remaining strains of *M. kingii* and the strains of *B. corrodens* were used only for immunodiffusion.

The methods were the same as described in the preceding paper. Sera against most of the antigens were prepared by both methods described in that paper.

RESULTS

The results of the agglutination tests are presented in Tables 1 to 4. Tables 1 to 3 show that heated antigens of most of the strains of *M. nonliquefaciens* and of the strain of *M. bovis*, like *M. kingii*, are agglutinated to higher titre than unheated antigens. This was also the case with the unclassified strain. This suggests that these strains may have a surface component which inhibits agglutination and which is removed by heating. The single strain of *M. lacunata* (with the homologous serum) and one strain of *M. phenylpyrouvica* did not show any change of agglutinability after heating.

One strain of *M. phenylpyrouvica* and the two strains of *M. osloensis* against which immune sera had been prepared, on the other hand showed a moderate or marked reduction of the titre after heating, suggesting destruction or deterioration of an agglutinogen by the heat.

The 4 strains of *Acinetobacter* showed variable behaviour. In one case agglutinability remained unchanged after heating, and in the remaining cases heating caused a marked reduction of the titre. Others have had similar experience with these organisms (*Stuart et al.* 1949, *Mannheim* 1962).

TABLF 1

Agglutination Reactions in Immune Sera against Moraxella phenvlpyrouvica *and an with Unheated (a) and Heated (b) Antigens*

Antigens	Immune sera					
	\ 7784		\-13385/12		\ 1797>	
	a	b	a	b	a	b
L-9833	160	640	–	–	80	320
B 9425	0	0	–		40	80
\ 7784	320	1280	40	0	160	640
\-826	0	0	0	0	80	40
\ 13385/62	40	640	40	1280	80	640
\ 672/58	0	160	0	640	40	1280
\ 4863/62	40	640	40	320	80	640
\-3898/60	0	0	0	160	80	640
\ 4378/62	40	640	0	640	80	640
\-17954	0	0	0	80	40	640
\ 3326	40	0	0	–	80	80
\-2916/61	–	640	–	40	–	160
\ 17975	40	160	0	80	160	2560
U-17955	0	20	–	80	0	80
P-2863	0	0	–	–	40	0
P 752/52	0	0			20	0

– not done B *M bovis* L *M lacunata* Lw *A lwoffi* A *A calcoaceticus* K *M kingii* \ *M nonliquefaciens* O *M osloensis* P *M phenylpyrouvica* U unclassified Moraxella, probaby *M phenylpyrouvica*

The strains O-41920 O-5873 O-8292 O-19116/51 O-10973 P-17958 A-75150 A 17912 Lw-17985 I -17977 and K-5530 gave entirely negative reactions

TABLF 2

Agglutination R t s in Immune Sera against M raxella phenvlpyr uvica *an l in Unclass (St a n with Unheated (a) an l Heat d (b) Antigens*

Antigens	Immune sera						
	U 179 5		P-2863		P 752/52	I 17958	
		b	a	b	a		b
I-9833	0	320	0	40	0	40	0
\-7784	0	320	0	80	0	0	40
\-13395/6	0	160	0	0	0	0	0
\-672/58	0	0	0	80	0	0	0
\-4863/62	0	160	0	0	0	0	0
\-4378/62	0	160	0	0	0	0	0
\-17954	0	40	0	40	0	0	0
\ 2916/61	0	160		40			0
U-17955	160	2 60	0	0	160	0	0
P 2863	0	0	10	160	0	40	0
P 752/5		0	–	0	1 0	–	0
P-17958	0	0	0	40	40	80	160
K-5530	0	0	0	40	0	0	0

Symbols as in T ble 1

These tra ns gave enti el negative re ctions O-41920 O 19116 51 O-10973 O-5873 A 15150 A 1791 I -17985 Lw -17977 B-9425 \ 826 \-3898 60 \-3326 and \-17975

TABLE 3

Agglutination Reactions in Immune Sera against Moraxella lacunata and M. bovis with Unheated (a) and Heated (b) Antigens

Antigen	Immune sera			
	L-9833		B-9425	
	a	b	a	b
L-9833	640	640	160	40
B-9425	0	0	40	320
N-7784	80	0	80	80
N-892	160	80	0	20
N-13385/62	40	0	0	320
N-672/58	0	0	0	320
N-4863/62	80	20	0	160
N-3828/60	0	20	0	160
N-4378/62	80	40	0	640
N-17954	0	0	0	40
N-3326	0	40	0	80
N-2916/61		40	–	40
N-17975	0	0	0	80
L-17955	0	40	0	80

Symbols as in Table 1
The following strains gave entirely negative reactions O-A1920 O-19116/51 O-10973 O-5873 A-35150 A-17912 Lw-17985 Lw-17977 P-2863 P-752/52 P-17985 and K-5530

Tables 1 and 3 show that cross reactions are very common within *M. nonliquefaciens* and between this species and *M. bovis* and *M. lacunata*. This suggests that many strains share or possess similar antigenic determinants and is in agreement with the view that these organisms are closely related. But more detailed studies of larger numbers of strains would be needed to clarify the serology of these species.

Three of the strains of *M. phenylpyruvica* gave weak cross reactions in some sera against the three just mentioned species and the latter also show some cross reactions in some sera against *M. phenylpyruvica*. But no signs of serological relationship of any of these strains with the strains of *M. osloensis* and with Acinetobacter was found. The strains of *M. osloensis* and of *M. phenylpyruvica* appear to be quite heterogeneous.

The few strains of *Acinetobacter* show marked mutual cross reactions in sera against one another but do not cross-react with any Moraxella strain. In view of the very limited number of strains these findings are of limited significance.

The results of immunodiffusion tests are shown in Table 5. It is seen that antigens prepared from the strains of *M. lacunata*, *M. bovis* and *M. nonliquefaciens* show marked cross reactions and appear to constitute a separate group. The strains of *A. calcoaceticus* and *A. lwoffi* also show marked although more irregular cross reactions. The antigens prepared from the three strains of *M. phenylpyruvica* produced only very few or no precipitate bands with the corresponding immune

TABLE 4

Agglutination Reactions in Immune Sera against Moraxella osloensis, Acinetobacter calcoaceticus *and* A. lwoffi *with Unheated (a) and Heated (b) Antigens*

Antigens	Immune sera											
	O-41990		O-10973		A-15150		A-17912		Lw-17985		Lw-17977	
	a	b	a	b	a	b	a	b	a	b	a	b
O 41990	>5120	320	0	160	0	0	0	0	0	0	0	0
O 10973	0	0	640	320	0	0	0	0	0	0	0	0
A 15150	0	0		0	160	320	0	0	0	0	0	0
A 17912	0	20		0	2560	0	>1280	>5120	0	0	>5120	40
A 17985	0	0		0	0	0	0	0	>1280	2560	0	160
Lw-17977	0	0		0	1280	10	5120-	160	0	0	>5120	640

Symbols as in Table 1

The following strains gave entirely negative reactions O-5873 O-19116/51 N 926 N-672/58 A-4863/62 A-3928/60 A-4378/62 33 F N 2916 60 A-7794 A-13385 A 17954 A 17975 P-752/52 P 2863 I 17958 U 17955 F 9833 B 9435 and K-5530

TABLE ,

Immunodiffusion Tests

Antigen	Immune sera															
	J 9833	B 9425	N 7784	N 17975	N 13385	I 2863	I 752	I 17958	I 179 5	K 5530	O 11990	O 10973	I 15150	I 14912	Iw 17985	Iw 17977
I 9833	3	3	2	3	9	0	0	0	1	0	0	1	0	0	2	1
B-9425	3	3	1	3	>	0	0	0	0	0	0	1	0	0	1	0
N-7784	1	3	5	3	3	0	0	0	1	0	0	2	0	0	0	0
N-17975	4	,	3		3	0	0	0	1	0	0	2	0	0	0	0
N-13385/63	2	3	3	4	3	0	0	0	1	0	—	0	—	—	—	—
I-2863	0	0	0	0	0	1	1	0	1	0	0	0	—	—	—	—
P-752/59	0	0	0	0	0	0	1	0	1	0	—	0	—	—	—	—
I 179 8	0	0	0	0	0	0	1	0	1	0		2	0	0	0	0
U-1795	0	1	0	1	1	0	>	0	5	0	0	2	0	0	0	0
K- 530	0	1	0	0	0	0	0	0	0	3	0	0	—	—	—	—
O-11990	3	1	1	—			~	—		0	5	0	0	0	>	0
O 10973	1	1	1	>	1	0	1	1	2	0	0	3	—	—	—	—
I-15150	1	0	1					—	—	0	0	0	3	0	9	1
I-17912	0	0	,		—			—	—	0	0	0	9	3	2	1
Iw-17985	0	0	1					—	—	0	0	0	0	0		0
Iw-17977	0	0	0		—			—	—	0	0	0	2	2	1	4

Figures indicate numbers of precipitate bands — = not done. Other symbols as in Table 1.
The serum O 10973 gave one precipitate band with antigens prepared from ? out of 11 strains of *M. lingu* and from 7 out of 11 strains of *B. corr lens*

sera Whether this is due to poor antigenicity or to failure of liberation of antigens by the ultrasonic treatment is uncertain but the latter alternative may seem more probable The two strains of *M osloensis* appear to produce mainly strain specific antibodies but the strain 0-10973 is characterized by rather wide cross reactivity both of the antigen in different immune sera and of the immune serum with different antigens The unclassified strain U 17955 shows a similar behaviour No explanation of this wide cross reactivity can be offered

DISCUSSION

The purpose of this study was to seek general orientation about the serological characteristics of Moraxella organisms and some organisms which have been thought to be related to Moraxella Although it was realized that many perhaps all the species studied probably were serologically inhomogeneous it was nevertheless decided to do a preliminary study of single or a few strains of each species

Whereas considerable information is available about the serology of *Acinetobacter calcoaceticus* (anitratus) and *A lwoffi* (*Stuart et al* 1949 *Ferguson & Roberts* 1950 *Mannheim* 1962 *Nelson & Shelton* 1965 *Friederichs et al* 1967) next to nothing was known about the serology of the oxidase positive Moraxella species Some Moraxella strains and some strains of *Mima polymorpha var oxidans* (which may or may not have represented known Moraxella species) have been included in serological studies of the so called *Mimeae* (*Cary* 1961 *Mitchell & Burrell* 1964) but the exact identity of most of these strains is unknown and the significance of these findings therefore is uncertain

It seemed natural to choose the agglutination test as one of the methods and to supplement it with gel diffusion tests with extracts of ultrasonically disrupted cells a method which has been used before (*Mitchell & Burrell* 1964) It seemed worth while to try to find out what this method would yield with strains of known identity

The results of the agglutination tests show that the strains of *M lacunata* (in heterologous immune sera) *M bovis* and *M nonliquefaciens* like the strains of *M kingii* studied in the preceding paper are agglutinated to a high titre after heating of the antigens The increase in most cases ranges from 4 fold to 32 fold This suggests that all these organisms have a thermolabile surface component which is itself nonantigenic or of low antigenicity and which inhibits agglutination The unclassified strain 17955 showed the same behaviour and it is worth noting that many heated but not unheated Moraxella antigens cross reacted with this serum This strain was received labelled *M nonliquefaciens* and in most characters it behaved like this s but it deaminated phenylalanin like *M phenylpyruvica* and

M nonliquefaciens Further studies are necessary to establish the identity of this strain

The strains of *M phenylpyrouvica* and of *M osloensis* are agglutinated to the same or a lower titre after than before heating In this they resemble the strains of *A calcoaceticus* and *A lwoffi* Three of the four strains were agglutinated to a lower titre after heating and the fourth strain was unchanged These strains therefore have a thermolabile surface antigen which is damaged or destroyed by heating It has been shown in previous studies (*Stuart et al* 1949 *Mannheim* 1962) that many strains of the two Acinetobacter species have a thermolabile surface antigen which causes the bacteria to be agglutinated to a very high titre and which is destroyed or damaged by heating Other antigens which *Mannheim* calls O antigens appear after heating to 100 C Autoclaving brings out still other antigens which Mannheim classifies as deep antigens (Tiefenantigene) whereas *Stuart et al* call them O antigens *A calcoaceticus* often appears in an encapsulated from which can be divided into numerous capsular types The relationship between the capsular antigens and the thermolabile surface antigens does not appear to be clear

The different behaviour of the classical Moraxella species (including the unclassified strain) on one hand and of *M phenylpyrouvica* *M osloensis* and the Acinetobacter strains on the other is interesting but difficult to interpret

The results of the agglutination tests demonstrate frequent cross reactivity but also marked heterogeneity between strains of the three classical Moraxella species The unclassified strain appears to be related to the same group Cross reactions with *M phenylpyrouvica* are few and weak and there are no cross reactions with *M osloensis* or the Acinetobacter strains These preliminary results suggest that it might be practicable to clarify the serology of the classical Moraxella species further but that inclusion of more strains and the use of absorption tests would be necessary

The strains of *M phenylpyrouvica* appear to be serologically heterogeneous likewise the strains of *M osloensis* The four Acinetobacter strains do not cross react with any of the other organisms In view of the known great heterogeneity within this group this finding is neither surprising nor very significant

The experience with the immunodiffusion method indicates that this is a very difficult method to standardize satisfactorily Even if a reasonable standardization of the bacterial suspensions is possible, the strains appear to vary considerably in sensitivity to ultrasound and different strains need different lengths of ultrasonic treatment to reach similar states of disintegration There is reason to suspect therefore that variations in the numbers of precipitate bands produced may at least in some instances rather be due to unsatisfactory standardization than to real serological differences Another serious limitation of the

method is that the nature of the antigens demonstrated is quite uncertain and the significance of positive reactions therefore impossible to assess If e g the reactions were due to protein antigens they might be highly significant Cross reactions between polysaccharide antigens on the other hand are so frequent and so often due to accidental structural similarities that their significance is very uncertain When these limitations are kept in mind it may be stated that some of the results obtained have a certain interest Thus the marked cross reactivity between all the strains of *M lacunata M bovis* and *M nonliquefaciens* is in very good agreement with the view that these organinisms are closely related

The strains of *M phenylpyrouvica* did not seem to be well suited for this kind of study Very few precipitate bands were produced probably suggesting unsatisfactory disintegration of the cells

Table 5 also shows that there are rather numerous cross reactions which are difficult to explain thus the numerous reactions of antigens from the strain 17955 in different immune sera and of other antigens in the immune serum against this strain and the similar cross reactions of strain 10973 and of its immune serum

Although serological studies of well defined bacterial antigens e g extracellular proteases have given very useful results the study of unknown antigens obtained by disruption of the cells appears to be much more difficult

SUMMARY

Serological studies were carried out with representative strains of species belonging to the genera *Moraxella* and *Acinetobacter*

Strains of *M lacunata*, *M bovis* and *M nonliquefaciens* showed marked cross reactivity in agglutination and immunodiffusion but signs of heterogeneity were also apparent

In the cases of *M phenylpyrouvica* and *M osloensis* mainly strain specific antigens were demonstrated There was no cross reaction between *Acinetobacter* strains and *Moraxella* strains in agglutination tests

In immunodiffusion a number of cross reactions were demonstrated which are difficult to explain The limitations of the method for such studies are discussed

Agglutination tests showed that the agglutinability of the strains of *M bovis* and *M nonliquefaciens*, like *M kingii* is increased by heating The agglutinability of *M phenylpyrouvica M osloensis and Acinetobacter* on the other hand was unaffected or reduced by heating

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